

# Baltimore defeat a defeat for research

Last week's resignation of Dr David Baltimore is a sad business. His obstinate defence of a research paper may have been the chief cause, but others must shoulder some of the responsibility.

DR David Baltimore's resignation as president of the Rockefeller University is, and should be, a matter of general regret. One of the most talented scientists of this generation, he has been brought low by an excess of pride. At no point has he been accused of personal dishonesty, as has one of the co-authors of the well-known and now-infamous *Cell* article on the immunology of transgenic mice. For much of the time since that appeared in April 1986, he could honourably have washed his hands of what was at best a muddy contribution to the literature.

Baltimore chose instead to stand by his colleagues from outwardly admirable, but quixotic, loyalty. That position would have been defensible if it had been based on a thorough examination of the data — and a thorough cross-examination of their authors, for which he would have been admirably suited. Incomprehensibly, it was not. That neglect was Baltimore's crucial error of judgement.

Others were his casuistical line of defence that the conclusions of the disputed article would and should stand or fall by others' confirmation of them, his frequent assertion that supporting evidence was, in any case, accumulating and his temporarily successful attempt to mobilize the scientific community in resistance to a supposed attack by the US Congress on freedom in science. Baltimore conducted a combative, even a brave, campaign. It is to be hoped that those who followed him do not generally share the opinions of the survivors of those whom Lord Cardigan led into the charge of the Light Brigade. In this case, there are no fatalities, but injured feelings may be the more damaging because those who hold them are alive.

For many, the turning point came this summer, with Baltimore's insensitive reply (*Nature* **353**, 9; 5 September 1991) to Professor Paul Doty's earlier direct criticism of his conduct. His letter did not meet the main charge, that senior authors have a first duty to the integrity of what they have published. Baltimore was warned that his letter would seem incomplete unless that complaint was dealt with, but he chose not to tackle it. Yet that issue is general in character, and separate from the rights and wrongs of the published article. The perversity of Baltimore's position is mystifying. How can a person hold that the stated conclusions of a scientific investigation are independent of the validity of the data by which they are purportedly sustained?

Many lessons will be drawn from this sad business

(which, sadly, is not yet ended). One concerns the conduct of academic institutions faced with accusations of misdemeanour. Both Tufts University and the Massachusetts Institute of Technology (MIT) set up committees to investigate Dr Margot O'Toole's criticisms of Dr Thereza Imanishi-Kari's work — the immunology part of the disputed article. Both were casual affairs.

Each inquiry was, in retrospect, thoroughly inadequate. Those concerned did not give sufficient weight to O'Toole's suspicions. That is the natural inclination of academics, who know how scandal can damage their institutions and that suspicions of misdemeanour may spring from ignorance or even mischievousness. But all institutions, not just Tufts and MIT, should now be more acutely aware that too casual an approach to questions such as these carries serious risks, not least that autonomy in their conduct of affairs will be ceded to quaintly named bodies such as the Office of Scientific Integrity (OSI) of the National Institutes of Health (NIH). Paradoxically, institutions will be rid of the present burden of misconduct cases only when they equip themselves to deal with emergent scandals vigilantly, openly and off their own bats.

NIH must also shoulder some of the burden of Baltimore's resignation. Too much time has passed since the disputed article appeared. NIH's first inquiry uncovered nothing remarkable. Why was that? And would the inquiry have been reopened if Congressman John Dingell had not insisted that it should be? But the controversy triggered by the leaking of OSI's draft report in the early summer and the subsequent uncertain reorganization of OSI have created a corrosive atmosphere that must have contributed to Baltimore's acknowledgement that he could no longer command the confidence of his academic colleagues. NIH now seem to follow others in saying that OSI's old procedures neglected due process, but there is no sign of when new procedures will be in place, or this inquiry be completed. Do not NIH know that justice delayed does not deserve the name?

Other institutions share in the responsibility. From the outset, Baltimore's defence of a second-rate research paper had the makings of a tragedy. So it has proved, not only for him, but for the public reputation of research. Even if the charges in the draft report are proved false, the research community will be blamed for taking so long to fail to get to the bottom of things, and for giving the



whistleblower in the case a rotten time. Where have been the academies and the learned societies during all the fuss? Where are they now, when the most urgent need is for a speedy conclusion to a still-corrosive issue? Complaints abound that Dingell is a bully, but do not the academies and societies understand that the role of the US Congress in policing the manners of the research community is partly a measure of their own neglect?

Baltimore himself now deserves the respite from controversy for which he asked last week, on behalf of his family especially. So, too, does the Rockefeller University, the vacillations of its trustees and faculty in the past few weeks notwithstanding. The best outcome would be what Baltimore hopes for himself — a return to research, perhaps in AIDS. There is nothing like creative work to chase bitterness away. It would help to that end if the Dingell Committee would quickly clear up the issues outstanding from the 1989 hearings. And those tempted to ask whether the punishment fits the crime should remember that there is no crime, but a gross error of judgement magnified by obstinacy into a public scandal and that there is hardly a punishment more severe than having to resign as leader of the institution at which one began a research career such as Baltimore's. □

## Poor rich Japan

There is little merit in US demands that Japan should contribute to the new accelerator in Texas.

POOR rich Japan! For the past several months, a succession of visitors from the United States have been in Tokyo pleading for what in other contexts is called technical assistance (and which may often consist of mere money) with the construction of the Superconducting Super Collider (SSC) now being built in Texas. Last week (see page 424), it was Admiral James Watkins, the Secretary of Energy. Next month, President George Bush, on a delayed visit, will raise the issue. The United States is looking for about \$1,000 million, in cash or in kind. Japanese officials have always made it plain that their government will say "Yes!" only to the US President himself. But, in everybody's interests, they should be steeling themselves to say "No!"

Not that international collaboration on accelerators at the frontiers of high-energy physics is a bad thing. On the contrary, as experience in Europe has shown, only collaboration can allow any but the richest parts of the research enterprise to build their own machines. Even in the United States, the high-energy physics community acknowledges that the time must come when joint construction will be unavoidable. But when? For decades, the received opinion has been that international collaboration will be indispensable for the accelerator after next.

What is being offered to Japan is not, in reality, collaboration, but an opportunity to contribute to a machine already fully designed and whose components are mostly fully developed. The argument that Japan would

thereby be placed at the frontiers of accelerator technology is mistaken; the mere replication of components already designed is no substitute for the execution of an advanced design. In return, Japanese physicists would presumably have access some of the data from the detectors, in the design of which some of them have had a hand. But that makes light of the boast in high-energy physics that hospitality at one another's machines is habitual.

But should not Japan be investing more in basic research? That is what the United States is also urging. Most Japanese would agree — and would point to the figures in the current research budget showing buoyant spending on basic research at universities and research institutes. Spending \$1,000 million on a machine in Texas will not advance that cause, but will, if anything, impede it. Even in rich Japan, the money would have to come from somewhere. And the consequences of that could be unfortunate. Already, resentment in Japan at what is bound to seem an attempt to dictate the pattern of another's research budget is too palpable for comfort. □

## Nature's Macmillan

Alive, Robert Maxwell embarrassed many, but the aftermath of his death is peripherally embarrassing for *Nature*.

THE dramatic collapse last week of the late Mr Robert Maxwell's publishing organization is a general embarrassment and also, strange as it may seem, for this journal. The difficulty is that Maxwell's Maxwell Communications Corporation owns a publishing company in the United States called "Macmillan" which is easily mistaken for the British publishing company of the same name, which happens to own *Nature* (which it founded).

Until after the Second World War, the US company was a subsidiary of the British. Before publishing became an international business, the British company sold its US subsidiary, with the understanding that the newly independent company could use the name "Macmillan" only in the United States; the British company retained that same right elsewhere in the world. (The British company afterwards set up a new subsidiary in the United States, which is now the successful St Martin's Press of New York, but *Nature*'s interests in the United States are managed by Nature Publishing Co. Inc., also of New York.) Until quite recently, the two companies coexisted amicably, despite the inconvenience of apparent identity.

Maxwell's purchase of US Macmillan (the cost of which, at \$1,300 million, appears to have helped push his companies into insolvency) changed that. The US company became careless about the covenants restricting its use of the common name, and Maxwell adopted a logo resembling that of the British company. Legal suits are said to have been multiplying. This journal's concern is that confusion over the common name should not lead subscribers or advertisers to conclude that we are on shaky ground. Our owners say they are both solvent and profitable. Moreover, the company's pension fund is intact. □



# FDA vs. free speech over drug promotions

- New rules aim to legitimize alternative drug uses
- Agency restricts conferences, press releases

## Washington

In a clash that pits the constitutional rights of industry against government responsibility to regulate drugs, the Food and Drug Administration (FDA) is battling the pharmaceutical industry over the promotion of products for uses other than those for which they were approved.

In recent months, FDA has cracked down on industry-sponsored scientific conferences and drug company press releases that the agency says are actually advertising aimed at promoting uses that have not been approved. Agency officials have angered the pharmaceutical industry by proposing rigid new guidelines to define what FDA considers an acceptable industry-sponsored scientific conference, rather than a cloaked promotion for a company's products (see sidebar).

This week, FDA commissioner David Kessler is expected to announce new rules he hopes will quell the controversy by allowing drug companies to circumvent the normal long approval process for alternative drug uses. What the agency has settled on, Kessler said last week, is a way to speed the transition from 'off-label' to approved status for secondary uses by reducing the amount of data and documentation a company must submit to gain

agency approval. "Once it's on the label," Kessler said, "you can promote it all you want." Kessler is expected to announce the new policy at a meeting on off-label drug uses this week at the Institute of Medicine.

Part of what pushed the FDA to take new measures is the explosion of industry-sponsored symposia. In 1988, the drug industry spent \$85.9 million on funding 34,688 scientific conferences. That, according to the Congressional Research Service, is more than a 13-fold increase from 1974.

In the past year, Kessler has doubled the size of the division that regulates marketing and has started to crack down on the industry. On 26 October, FDA issued a draft 'concept paper' listing 20 criteria a scientific conference should fulfil to be considered 'independent'. FDA also wrote to pharmaceutical companies, telling them to send the agency copies of all their press releases and 'video news releases', short videotapes that many companies produce and send to television stations to promote their products.

Now, FDA is itself facing scrutiny, as critics charge that the agency's new rules deprive companies of their constitutional right to free speech. "There is a real feeling

of paranoia out here," says Susan Sauer, director of communications for the pharmaceutical company Sigma Tau. To ensure that the FDA does not accuse them of a violation for undue promotion of unapproved drug uses, some companies have taken to sending FDA drafts of the press packages for approval before release. Other companies are just not talking. One reporter recalls a recent case in which drug company researchers stopped cooperating on a story about a new drug use, fearing that the FDA would see their quotes and call it promotion.

Kessler said last week that he is not going to back down on restricting drug promotions if they go beyond approved uses. Particularly in cancer treatment, many drugs are approved for a single disease, although they are often also prescribed for other, related conditions, a practice known as 'off-label' use. Acne drug Retin-A gained international attention several years ago when it was promoted as an off-label way to reduce wrinkles. Although FDA cracked down on that use, arguing that there was no evidence it was effective, officials estimate that some 20 per cent of off-label uses are actually the best available therapy for the relevant condition. Doctors may freely prescribe drugs for such secondary uses, although many insurance companies often refuse reimbursement for off-label drug prescriptions.

Although FDA can do nothing about the off-label drug prescriptions, it can stop drug companies from promoting such use in the absence of data to show its efficacy. "If you can promote the secondary use without approval," Kessler asked, "where's your incentive to do the testing and the [clinical] trials? The problem we have here is one of hype."

But with the pharmaceutical industry up in arms, FDA decided to find a compromise that does not sacrifice public health for industry's right to free speech. According to William Hubbard, FDA's assistant commissioner for policy coordination, the new rules will allow companies to submit journal articles, unpublished clinical trial data and any other evidence they may have acquired to suggest that an approved drug may have another effective use. At the moment, drug companies are required to go through the same elaborate approval process for secondary uses as for primary use, something that few were willing to do, considering that they could sell the drugs without it.

"We believe that companies won't have to go out and get new data," Hubbard says. "The fact that an [off-label] use is popular means that the data are out there." He says that the agency hopes to have all the major unapproved cancer uses "on the label" within a year, by encouraging the companies to come in from the cold, bearing any data they happen to have.

**Christopher Anderson**

## When is a conference independent?

### Washington

In seeking to ensure that industry-sponsored scientific conferences are not advertising campaigns in disguise, the FDA is preparing to issue some tough new rules on what is, and is not, an 'independent' symposium. An FDA 'concept paper' issued on 26 October proposed some rigid new definitions of what FDA considers science, rather than salesmanship.

To escape sanctions, FDA says that industry-sponsored conferences should:

- include independent experts. "The drug company should play no role in the selection of presenters or authors."
- disclose their finances. Organizers should reveal "the actual source of funding ... and relationships between individual presenters and the drug company."
- not focus on a specific drug. Conferences "should be on broad aspects of a disease and on a variety of treatments."
- separate the sponsors from the sub-

stance. The industry sponsor should agree not to engage in "scripting, ghost-writing of papers, preparation of visual aids, training of presenters, or targeting of points for emphasis." Nor should there be any promotion, such as distribution of material, "in proximity to educational activity".

■ not be repeated. Holding a conference more than once "enables drug companies to judge content and to selectively support repeat presentation only of programmes favourable to its product."

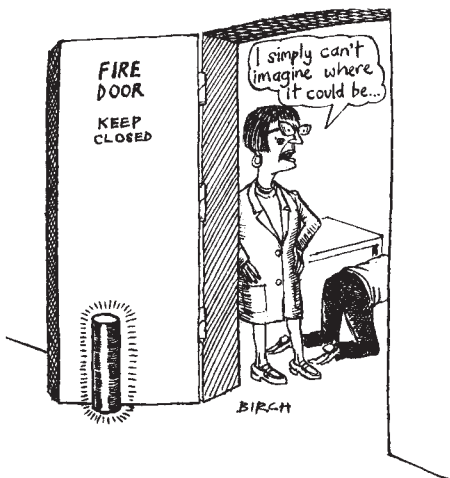
Not surprisingly, the Pharmaceutical Manufacturers Association (PMA) is up in arms over the proposed rules. It said that the rules, if adopted, would "severely and unnecessarily limit the continued and timely dissemination of medical and scientific information". By listing 20 "essential" criteria for an acceptably independent conference, the FDA concept paper "goes well beyond what is appropriate and reasonable." **C.A.**



# Dounreay loses uranium

## London

ONE of the two nuclear waste reprocessing facilities at the UK Atomic Energy Authority (AEA)'s plant in Dounreay, Scotland, has been shut down after plant managers found that they cannot account for



some 10 kg of enriched uranium. The discrepancy may be simply due to an error in accounting — no leaks of radioactive material have been detected, and plant security should prevent anyone removing uranium from the site — but the revelation

is highly embarrassing for AEA. Enriched uranium can be used in weapons production, and the 'loss' of a substantial quantity emerged last week at a time when inspectors from Euratom, the European Atomic Energy Community, were visiting Dounreay.

The plant handles spent fuel from AEA's prototype fast reactor, also at Dounreay, and provides a commercial reprocessing service for a number of smaller research reactors. Because of the difficulty in measuring precisely the quantity of uranium passing through the plant, AEA assumes that no uranium has been lost if the quantity measured after reprocessing is within 2 per cent of the amount that should be present. For security reasons, AEA will not reveal the amount by which the unaccounted 10 kg of uranium exceeds this margin of error, but a spokesman says the discrepancy is "much larger" than that usually allowed.

The offending section of the plant will remain closed until a team from AEA's London headquarters, led by nuclear materials accountancy expert Wyn Llewelyn, has solved the mystery of the missing uranium. AEA promises a public report once Llewelyn's findings are known.

**Peter Aldhous**

PLAGIARISM

# Misconduct increases at NSF

## Washington

SCIENTIFIC misconduct cases being investigated by the National Science Foundation (NSF) have risen more than 16-fold in the past two years, the agency's inspector general reported last week. At the end of 1989, NSF was investigating three misconduct cases; it now has 49 cases in progress and has been forced to add more staff to work exclusively on misconduct.

Two of the most serious cases highlighted in the inspector general's report involve plagiarism, a charge that is "very common among the misconduct cases we receive", the report says.

In one of the cases, a researcher in the electrical engineering department of a "large midwestern university" (NSF did not identify the parties in any of the cases) copied extensively from two other researchers when preparing his grant applications. Responding to a university investigative panel, the researcher claimed that although he might have "mistakenly" copied text from two papers, his proposed methods were novel. But after inspecting the works, the panel concluded that the researcher's "unique contribution" was "linking one source's introduction and definition of the problem to another source's presentation of the method of

solution." The inspector general recommended that the researcher be barred from receiving federal funding for three years.

In the other case, an agricultural researcher in a "small southern university" copied the section on research methods in his NSF grant application from another paper. Again, when challenged, the researcher used a 'carelessness defense'. The university investigative panel accepted the excuse and concluded that the researcher had "seriously deviated from accepted practices", but had not committed plagiarism.

This defence, the NSF report argues, is a red herring, but an increasingly common one. "We have found that questions about the subject's intent frequently arise in inquiries and ... often introduce confusion." NSF takes the position that by signing grant applications, researchers take responsibility for any plagiarism found in them, regardless of intent. "Inquiries into the state of mind of those researchers are beside the point in situations where express certification is provided," the NSF report concludes. In this case, the inspector general disagreed with the university investigative panel, found plagiarism, and recommended a two-year debarment.

**Christopher Anderson**

# Logjam ahead for UK academics

## London

THE average Mexican academic has better access to supercomputers than his counterpart in Britain, according to a new report from the Parliamentary Office of Science and Technology (POST), the fledgling British answer to the US Congress's Office of Technology Assessment. Although in 1985 Britain's academic supercomputing facilities compared favourably with those in other developed countries, British investment has since lagged far behind that in the United States, Germany, Japan and Canada.

POST warns that the three British academic supercomputing centres — at the Science and Engineering Research Council's Rutherford Appleton Laboratory, and in university centres at London and Manchester — are already fully occupied, and that the Natural Environment Research Council (NERC)'s planned work on ocean and atmospheric circulation modelling will soon overwhelm the system. The dearth of supercomputers also brings a commercial penalty: British academics are increasingly turning to supercomputing centres in the United States, where they must make their results available to all US researchers. POST notes that results from UK government-funded research at University College London and the University of Swansea are now freely available to US aerospace companies.

If the projected supercomputing needs of British academics are to be met, the POST report says, then the government will have to spend substantially more than the extra £10 million it plans for academic supercomputing over the next two years. Providing NERC with its own machine, costing around £6 million, may be one answer to the problem, according to POST.

But costs could be cut by allowing academic researchers to work on the half-dozen supercomputers in Britain's defence research laboratories and the Cray-2 machine at the UK Atomic Energy Authority (AEA)'s Harwell laboratory. These are thought to have enough spare capacity to add a third to Britain's academic supercomputing capacity, but there are obstacles to their use for academic research projects. Michael Schomberg, computing manager at Harwell, explains that AEA is given rigid profit targets by the Department of Energy, and cannot allow academics free use of Harwell's machine. Nevertheless, Schomberg believes it should be possible for the Department of Education and Science to negotiate with the Ministry of Defence and the Department of Energy to find a way to improve academic supercomputing provision at a cheaper cost than buying new machines.

**Peter Aldhous**

# HDTV off to slow start

## Tokyo

JAPAN took a step ahead of the rest of the world in the development of high-definition television (HDTV) on 26 November with the start of regular HDTV broadcasts via satellite. Japan's HDTV has crystal-clear pictures that contain twice as many lines as conventional television. But most Japanese viewers of the new HDTV channel will probably not see the high-quality pictures because they will tune in on modified conventional television sets rather than HDTV sets, and the future of Japan's HDTV is uncertain.

The Japanese government, the National Broadcasting Corporation (NHK) and industry have invested more than \$1,000 million over 20 years in developing HDTV. But with the start of the new HDTV broadcasts, Japanese electronic companies are not trying to sell consumers HDTVs, which currently cost about \$30,000 each. Instead, they are promoting sales of much cheaper — yet still expensive — conventional televisions that have been modified to receive and display the HDTV signals.

The modified televisions are equipped with converters that translate the complex HDTV signals into a form an ordinary television can deal with. Some have wide screens that can display the full wide-angle HDTV picture. But the cheapest versions, priced at about half a million yen (\$3,800), have only slightly widened screens that cut off the HDTV picture at the sides. Nevertheless, these seem to be the ones in most demand.

Japanese manufacturers are rushing to bring down the price of real HDTVs by developing cheaper sets of semiconductor chips for the televisions. HDTVs require a high-performance computer, called a decoder, that reads and translates the vast amount of information contained in HDTV broadcasts. Japanese companies have teamed up with US manufacturers to de-

velop the new chip sets, and cheaper second-generation decoders should be on the market late next year. Industry's ultimate goal is to bring the price of HDTVs down to ¥1 million (\$7,600) in time for the Atlanta Olympic games in 1996, by which time they hope about 5 per cent of Japan's 40 million homes will have HDTV.

But many industry observers question whether consumers actually want to see high definition pictures at that sort of price. "Twenty years ago, NHK decided people wanted more lines on their TV screens, but what they actually want is more choice", says Bob Johnstone, Technology Correspondent for the *Far Eastern Economic Review*.

Johnstone and many other industry observers believe the future of television lies in a much wider choice of channels combined with new computerized televisions that will allow viewers to interact much more with their televisions.

Even if HDTV takes off, it is questionable whether it will be based on the system currently in use in Japan. The present Japanese system broadcasts analog signals just as does conventional television. But last year, the US company General Instrument announced development of a compression technology that the US company claims will allow transmission of HDTV signals in digital form, and most people in industry now believe that HDTV of the near future will be digital.

But this does not mean that Japan will be leapfrogged by the United States, as some observers have suggested. All of Japan's major electronic manufacturers are now working on development of digital HDTV, and the Ministry of Posts and Telecommunications plans to launch a Communications and Broadcasting Engineering Test Satellite (COMETS) in early 1997 that will test digital HDTV broadcasts.

**David Swinbanks**

## BIOTECHNOLOGY

# Synergie in Quebec

## Quebec

In an attempt to increase the innovative technological capabilities of its university researchers and industries — and thus the province's competitiveness in international markets — the Quebec government has set up a programme called Synergie to encourage university/industry collaboration. It will distribute \$32 million to successful applicants over the next five years.

Synergie will support projects that could help industry to produce new products or processes and commercialize them; that involve a level of risk a firm could not assume alone; that stimulate interest in innovation and technological development

in research centres or industrial firms; or that promote development of the personnel required for innovation.

Researchers in universities, colleges and affiliated organizations can apply. Their projects must involve at least two participating Quebec companies, must cost more than \$1.5 million and must last from one to five years. Companies must put up between 10 and 40 per cent of the cost, depending on their size.

The programme will be administered jointly by the departments of industry and higher education and science, and the first group of approved projects will be announced next March. **David Spurgeon**

# Canada targets biotech

## Montreal

ALTHOUGH Canada has not been as quick as other nations to recognize the importance of biotechnology to industrial growth, that situation is now changing. In recent weeks, the federal government has released a report aimed at strengthening the Canadian biotechnology industry, and both Canada's science minister and the president of the National Research Council (NRC) have taken up the issue.

*National Biotechnology Business Strategy: Capturing Competitive Advantage for Canada* finds a variety of obstacles hampering Canadian biotechnology development, including a lack of risk capital, trained managers and technicians, delays and uncertainties in governmental regulatory procedures, and a patent system that has produced a backlog of nearly 2,500 patents pending from as far back as 1979. At the rate patents are being awarded, the backlog would take 50 years to clear.

The report's recommendations include:

- creating funding pools of \$35 million to \$50 million for small-company investment, offering investors tax incentives and encouraging joint industry-provincial development funds;
- setting up high-technology company management courses, and making immigration easier for foreigners with required skills;
- upgrading university research through support of indirect costs; and
- streamlining regulations and patent laws and harmonizing them internally and with those of other countries.

Science Minister William Winegard told a press conference on 25 November that Canada has the brains to develop a world-class biotechnology sector, "but we haven't had the will or the way to clear some of the debris out of the way." He lauded the report and urged changes in industry's attitudes.

Nine days later, NRC president Pierre O. Perron told the agency's 6th Industrial Biotechnology Conference in Montreal that, after a year's thorough examination of its own biotechnology programme by another group of outside experts, "NRC is preparing a strategic plan for its long-term commitments in this area".

The outside evaluation of this programme took place as NRC celebrates its 75th anniversary. Perron said it was done at a time when the agency's activities are moving from laying biotechnology's foundations to a "second phase" where industry is commercializing its products.

More than 200 Canadian companies already use biotechnology, but the report identified four areas in which significant market opportunities are matched by Canadian strengths: waste management, forestry, food and agriculture, and human biopharmaceuticals. **David Spurgeon**





## Poles together

### London

FRANCE's polar research programme will next year be brought under one roof, with the opening of a new institute in Paris to coordinate the work of all French polar researchers. For Claude Lorius of the Centre National de la Recherche Scientifique (CNRS)'s Laboratory of Glaciology in Grenoble, who will be the institute's president, last week's announcement of a FF113-million (about \$21 million) 1992 budget for the institute ends nearly two years of uncertainty. First proposed in February 1990, the institute's future became bogged down in negotiations between the ministry for overseas territories (responsible for France's interests in Antarctica) and the ministries of research and finance.

Lorius expects that the new institute's main effect will be on French research in the Arctic — which includes ice-core drilling in Greenland, the French contribution to a pan-European study of ozone depletion, and studies of indigenous Inuit people.

Until now, there has been no single body responsible for running these projects as a coherent Arctic research programme.

But France's Antarctic research effort, already one of the largest in the continent, should also benefit. The programme is now run by two organizations: Terres Australes et Antarctiques Françaises (TAAF) — an offshoot of the ministry for overseas territories — which finances the Antarctic programme, and Expéditions Polaires Françaises (EPF), responsible for running the programme in the field. Lorius says that the division of responsibilities between the two bodies has been unclear (see *Nature* 350, 299; 28 March 1991).

The new institute will supersede EPF and will leave TAAF responsible for only the logistical support of the programme. The centralized approach does not extend to French polar researchers themselves, however, who will remain spread among university and CNRS laboratories throughout France.

**Peter Aldhous**

### YUGOSLAV CRISIS

## Academics invade Dubrovnik

### London

THE Croatian Academy of Sciences and Arts is planning a brave — some would say foolhardy — expedition to focus attention on the destruction of Dubrovnik by the surrounding Yugoslav and Serbian forces. Next Saturday (14 December), academy president Ivan Supek plans to sail from the Italian port of Bari, through the Yugoslav navy blockade, and into the besieged port itself. With him, he hopes, will be some 50 academics, half of them from abroad. If allowed through the naval blockade, Supek intends to survey the damage to the city before sailing back to Italy and appealing for an end to the attacks on one of Croatia's most important

cultural and scientific centres.

Supek, a theoretical physicist turned novelist who fought with the Yugoslav partisans during the Second World War, says he had hoped to hold a conference in Dubrovnik's Interuniversity Centre. But this institute, an international centre run by a consortium of more than 200 universities, has been largely destroyed by fire, after being hit during the bombardment of the city.

Despite the obvious danger, Supek is convinced that academics will join his expedition. He says that a number of foreign academics have already agreed to take part, but declines to name them in advance of the sailing. **Peter Aldhous**

### HIV INFECTION

## France will compensate

### London

BOWING to public and media pressure, the French government will provide compensation for some 7,000 French citizens who have been infected with human immunodeficiency virus (HIV) through blood products and transfusions. Bruno de Langre, president of the French Haemophilia Association, which has been representing more than 1,000 infected haemophiliacs, says that a bill now passing through the French parliament will free claimants from the need to prove in court that the transfusion service was at fault. French insurance companies are expected to provide FF1,200 million (about

\$220 million) to a new foundation that will handle the compensation claims, but the French taxpayer will bear most of the burden. If all claimants are to be given full compensation, says de Langre, the total bill could reach FF12,000 million.

The French plans greatly exceed the compensation on offer in other countries. But the French government is fighting a low rating in public opinion polls, and allegations that the health ministry failed in 1985 to prevent the distribution of HIV-infected blood products have precipitated a major scandal (see *Nature* 353, 781; 31 October 1991 & 354, 98; 14 November 1991).

**Peter Aldhous**

## 3,000 lecturers wanted

### Hong Kong

A DESPERATE shortage of college and university lecturers in Hong Kong has led seven government-funded tertiary institutions to combine forces in a search for academic staff overseas.

The shortage stems from planned expansion of the territory's tertiary education system, which has the aim of doubling the number of first-year students by 1995. The result is a need for roughly 3,000 more lecturers by 1995, nearly the number of lecturers already working in Hong Kong's higher education system. Although the precise number of lecturers required in science and engineering has not yet been announced, large numbers will be needed to fill slots in the new University of Science and Technology and a new engineering department at the Chinese University of Hong Kong.

Because the institutions have a gentleman's agreement not to poach staff from each other, they have agreed to form a joint recruitment committee to seek faculty from overseas. The campaign, which began in late November, first goes to North America, with exhibitions in Toronto, Chicago and San Francisco, to which local academics were invited, and then it moves to the United Kingdom. If the two trips go well, the committee plans to also visit Australia and Singapore next year.

Institutions on the committee, in addition to Chinese University, are City Polytechnic of Hong Kong, Hong Kong Baptist College, Hong Kong Polytechnic, the Hong Kong University of Science and Technology, Lignan College and the University of Hong Kong.

Peter Gwynne

## \$25 million for R&D

### Hong Kong

HONG KONG plans to spend HK\$200 million (about \$25 million) on industrial research and development (R&D) projects next year. The amount represents a down payment on a new scheme to encourage applied R&D by a government that has previously taken a strictly *laissez faire* attitude to support of technology.

The requested allocation, which the finance committee will take up in December, comes in reaction to fears that Hong Kong is falling behind other Asian nations in its development of technological products for export. The grants are intended to encourage more private sector investment in applied R&D, said Tyebjee Barma, director-general of the industry department. "In return, the government could share in the benefits from commercially viable projects it has supported."

Over the next three years, Barma said, he hopes to increase the programme to at least \$50 million a year.

Peter Gwynne

# Human genome dispute

SIR — Your article "Secrecy and the bottom line" (*Nature* 354, 96; 1991) seriously misrepresents the Medical Research Council (MRC) in a number of respects. May I try to set the record straight?

The MRC is accused of secrecy with respect to the identity of sequences of fragments of cDNA. Before the decision of the Office of Technology Transfer at the National Institutes of Health (NIH) to file patents on its cDNA sequences, MRC intended to publish openly its sequences in its database. That remains MRC's preferred position. MRC believes that filing patents on cDNA sequences is counterproductive to the interest of industry and would not facilitate the delivery of long-term health care benefits, a view also stated by representatives of industry. However, following the patent application by NIH, we feel compelled to retain our option to file patents; the temporary withholding of the data was intended only to allow us time to react to the NIH initiative.

A second major misrepresentation is embodied in "holding its sequences secret while it prepares to sell them to industry". Access to the database is free to academics on the understanding that they provide information back into the database, as they generate pertinent facts. No such obligation is placed on industry which instead is asked to pay a modest subscription towards costs. This relationship between provision of service and charge is completely consistent with government policy. Reaction from industry to our proposals for a subscription scheme — both in principle and at the levels of charging imposed — has been

warm and has encouraged us to continue.

D. A. REES  
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■ The word "misrepresentation" hardly seems appropriate, for there is no discordance on facts. MRC may have intended to make its cDNA data freely available to academics, but had not done so in mid-November (and will not now do so until the patent issue is resolved); is a "secret" to be called something else if its perpetrators intend that it should eventually be something else? And nothing in the News article complained of was intended to suggest that MRC is acting outside the framework of (British) government policy, but merely that that policy may not be in the best interests of science and discovery. — Editor, *Nature*.

SIR — Contrary to your remark (*Nature* 354, 171; 1991), HUGO (the Human Genome Organisation) is very much alive. Established by the community of genome scientists three years ago, HUGO's central concern has been the coordination of scientific activities at the level of both single chromosomes and the whole genome.

A research project involving thousands of researchers around the world and generating enormous amounts of data poses immense problems of communication. Following Human Gene Mapping (HGM) Workshop 11 in London this summer, there is now clear agreement on the way forward. The scale of the task means that it is sensible to work on each chromosome separately, by means of a series of 'Single Chromosome Workshops'. HUGO is coordinating the organization of these meetings in location and geographical spread, timing and funding mechanism. Our guidelines should help with the planning and running of these workshops, particularly when common procedures can save time and effort.

We also plan annual Chromosome Coordination Meetings (CCMs) bringing together small numbers of people from groups concerned with each chromosome to discuss common problems and policies. The 1992 CCM will be in Baltimore, Maryland, and HUGO has just agreed with the Japanese genome community that it will host CCM93.

Every other year, the CCMs will be associated with restyled Human Genome Mapping Workshops, with wide interdisciplinary participation, allowing coordination of information on mapping and sequencing, assessment of overall progress on the Human Genome Project and discussion of the broader biological ques-

tions it will provoke.

In support of these developments, HUGO is concerned with the development and management of databases, DNA clones, cell lines and other biological reagents with the aim (among others) of ensuring that the availability of materials and the arrangements for their distribution are adequate. We are also compiling central registers of information about genome meetings, databases, projects, DNA collections and cell lines, and are beginning to act as a clearing-house for this material.

On the wider front, the recent appointment of Nancy Wexler and Alain Pompidou as co-chairs of an ethics committee signals HUGO's readiness to be the focus for international discussions of the social, legal and ethical issues arising. Meanwhile, HUGO's development of an international research programme on Human Genetic Diversity reflects interest in the functional significance of the data that will be generated.

You may be interested that the specific issues discussed in your article were first raised at a meeting convened by HUGO in August this year, during HGM11, when there was a frank discussion between representatives of most cDNA groups and others about the storage of and access to cDNA sequence data. HUGO is concerned that if the NIH patent applications for cDNA sequences are approved, scientific exchange will be harmed and the transfer of important technology for the benefit of human health will be impeded. We do not, of course, oppose the patenting of genetic information whose utility has been demonstrated, but rather the patenting of short sequences from randomly isolated portions of a gene encoding a protein of unknown function.

Apart from the difficulty of deciding on the novelty and utility of such patent applications, it seems likely that approval of them would cause competition between laboratories to escalate, discourage the sharing of information and promote a climate of genome 'ownership', all of which conflict with HUGO's aim of fostering international collaboration so as to avoid needless competition and duplication. We hope that governments will pay attention to these issues, and that they will develop patent policies that, while allowing developments based on the use of sequences to be appropriately protected, will permit the widespread sharing of materials and information and not jeopardize the Human Genome Project as a major international collaboration.

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## Playing the game

SIR — Christopher Anderson, in his report of Ohio State University's decision to withdraw from the Columbus telescope project (*Nature* 353, 97; 1991), said the announcement came as Ohio State's (American) football team arrived in Tucson for a game and suggested that, to make matters worse, "the Ohio State team . . . trounced the Arizona Wildcats, 38-14".

Is *Nature* suggesting that the outcome of that game (which took place incidentally in Columbus, Ohio, not in Tucson, Arizona) was or should be linked to a decision on the future of academic research, or that the gentlemanly thing to do would have been to lose the game?

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# National publication bias

SIR — The availability on disk of *Current Contents* makes possible a variety of statistical analyses of publications. The letter on national publication bias in scientific journals from E. Ernst and T. Kierbacher (*Nature* 352, 560; 1991) prompts me to report what may be called a 'language bias'. The number of publications originating from selected major countries was searched in issues 18–23 of *Current Contents Life Science* volume 34, covering 600 journals. The United States as such was omitted because the papers of that country appear under each state name, but three major states (California, New York and Texas) were searched for comparative purpose.

The table shows that in the period examined, the United Kingdom (sum of England, Scotland, Wales and Northern Ireland) produced approximately the same number of publications as Japan and West Germany combined. It is also seen that Canada produced approximately as many publications as West Germany.

One would expect that countries with comparable gross national products (GNP) per capita would produce a similar number of publications per head (see table). The differences between the countries became even more striking: the United Kingdom produced as many papers per capita as Japan, West Germany and France together. Interestingly, the countries (or states) producing the most publications per capita were English-speaking countries (or with a majority of native English-speaking inhabitants). The only exception is Sweden.

It is suggested that this bias originates from the fact that the vast majority of the publications in the scientific literature are in English (the excellent English of Swedes is well known). The usefulness of a common language for science is not questioned, and English has proved

to be that. But writing in English can be a considerable burden to those not fluent in that language, which could partly explain the bias (science policies and evaluations systems are certainly contributing too). In addition, the bias described above has certainly some other effects. For example, because publications have to be written in good English, the editorial boards of many international high impact journals are strongly dominated by scientists from English-speaking countries. Another example is with the evaluation of research groups from different countries when competing for international grants (for example in the European Communities).

It would be interesting to examine the average impact of publications from English vs. non-English-speaking countries to see if the bias persists.

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## Tissue shortage

SIR — The lack of human tissue for research and education, a problem again identified by Christopher Anderson's report "Beating the tissue shortage" (*Nature* 351, 195; 1991), has prompted animal welfare organizations in Britain and the United States to launch the Humane Research Donor Card (HRDC), with carriers requesting that "after my death any part of my body be used for medical and scientific research". The HRDC does not preclude people from also becoming donors for transplantation, and they can carry both types of card.

The card is an initiative of animal protection groups because human tissue is very much a neglected alternative to laboratory animals. In pharmacology for instance, the use of human material is still the exception rather than the rule, despite the limited relevance of most animal tissue studies<sup>1,2</sup>. In fact, analysis of papers presented at the April 1990 meeting of the British Pharmacology Society indicated that only a small proportion of experiments using isolated tissues employed material of human origin — 84 per cent used tissue from animals, mainly rats and guinea pigs.

Since February 1991, when the British group Animal Aid first launched the HRDC, public response has been overwhelmingly positive, with more than 60,000 cards already distributed, indicating a genuine desire to help both medical science and animals.

Many scientists have also welcomed the scheme<sup>3</sup> and not only for humane

reasons: work with human tissues overcomes the problem of species variation and produces results directly relevant to people<sup>1,4-6</sup>.

Nevertheless, to achieve its full potential, there needs to be well co-ordinated systems of tissue storage, with hospitals and research institutions carrying a list of intended donors, thereby reducing the chance of tissues going to waste. If people carry the HRDC and inform relatives of their wishes, the problems encountered in Philadelphia will be avoided.

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## Designer data

SIR — Your issue of 26 September (*Nature* 353, 294; 1991) illustrates a 1989 West German stamp showing a population pyramid for the German population for the year "1989" and a projected population pyramid for the year 2000. These two pyramids are incompatible, because, although they are ostensibly based on populations 11 years apart, scrutiny shows that they are actually 15 years apart (for example, the peaks at age 20, 45 and 55 in "1989" correspond to ages 35, 60 and 70 in 2000). Which then is correct? The two conspicuous troughs in the "1989" distribution, at ages 40 and 67, seemingly result from the birth cohorts of 1949 and 1922. It is more likely that this is the pyramid for 1985, 15 years before 2000, so that the two troughs correspond to cohorts with depressed birth rates at the ends of two world wars, in 1918 and 1945.

I conjecture that the stamp's designer had been supplied with demographic data for 1985, probably the most recent year for which accurate statistics were available. Knowing that the stamp was to be issued in 1989, to commemorate the centenary of the social security system, the designer changed the date (but not the data) to correspond to 1989.

Scientific data are rarely printed on stamps; when they are, then aesthetics should be less important than accuracy.

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PUBLICATIONS IN SELECTED COUNTRIES OR STATES

| Country (or state) | Total number of publications | Publications per million inhabitants |
|--------------------|------------------------------|--------------------------------------|
| California         | 1274                         | 62.1                                 |
| New York           | 858                          | 47.1                                 |
| Texas              | 488                          | 43.5                                 |
| UK                 | 2268                         | 40.5                                 |
| Canada             | 895                          | 38.9                                 |
| Sweden             | 315                          | 38.0                                 |
| Australia          | 440                          | 31.0                                 |
| France             | 887                          | 16.6                                 |
| West Germany       | 901                          | 11.5                                 |
| Japan              | 1311                         | 11.4                                 |
| Italy              | 544                          | 9.6                                  |

Found in Vol. 34 issues 18–23 of *Current Contents Life Science* (600 journals).



# Leaking gas in the greenhouse

Max K. Wallis

Greenhouse gas emissions by the United Kingdom could be significantly reduced by replacement of old and leaking gas mains. Such a programme could even be cost-effective for the utility concerned.

BRITISH Gas plc, the newly privatized utility, estimates leakages of natural gas from distribution mains and service pipes, principally from aged cast-iron mains, to be less than 1%. But others<sup>1</sup> estimate leakage to be up to 11% of total supply. If leakage were as high as several per cent, the value of the lost gas means that replacement of the pipes could be financially advantageous. Because methane in the atmosphere is a potent greenhouse gas<sup>2</sup> it is important to account for leakages when calculating the comparative greenhouse effects of competing energy technologies.

Pipes laid before 1969 to carry town gas (mainly carbon monoxide) are expected to be several times leakier than post-1969 pipes designed to retain natural gas (methane)<sup>1</sup>. In Britain, there are 5 million service pipes (of a total of 17 million) and 140,000 km of mains (of 240,000 km) still in use from 1969 or earlier. These pipes leak badly because the hemp-lead joints dry out in methane. Efforts to recondition the gas have been partially successful for medium-pressure mains (operating up to pressures of 2 bar) but hardly effective for low-pressure distribution mains (about 30 mbar). Leakage via pipe breaks has been tackled by an enhanced detection and repair programme, presumed to be 80–90% effective (serious explosions have been cut from about 20 per yr in the 1970s to 4 per yr<sup>3</sup>).

To calculate leakage rates, I adopt the 'median case' assumption of Mitchell *et al.*<sup>1</sup> to derive the figures in the table. Although there is uncertainty to within a factor of 2, Mitchell *et al.* believe this case is 'conservative' in the sense of underestimating the real leakage rate.

I estimate the cost of relaying new mains to average £20 per m for low-pressure and £35 for medium-pressure mains, to be consistent with the £135 million spent in 1989 on laying 4,667 miles of mains (59% new mains, 85% low-pressure). I also take renewal of a service pipe to cost £85, consistent with the £45 million spent on 356,000 renewals and similar numbers of new services, mostly to new housing, at £40 each. British Gas assumes that new mains depreciate over 55 yr and service pipes over 35 yr. At 10% discount rate and a value of gas (to the company) of 20 p per therm (ref. 4), replacement of a service pipe saving 50.5 therms per yr (table)

results in an average net saving of £10.10 – £8.76 = £1.34 per yr while replacement of low- or medium-pressure mains (saving 4,500 or 5,800 therms per km per yr) results in a net cost of £1,100 or £2,350 per km per yr. Because of substantial variation in relaying costs and leakage rates, there may be cases where replacement of mains gives net saving.

From these average figures, one can calculate costs of saving greenhouse gas emissions in terms of tonnes CDE (carbon dioxide equivalent; 1 therm of methane weighs 2.0 kg). I adopt an equivalence factor between methane and CO<sub>2</sub> per unit mass of 50, uncertain to a

nuclear reactors<sup>4</sup>.

Prices quoted by British Gas for contract work on pipe relaying in 1991 are higher than used above, from £25 per m for the smallest to £80 per m for 200-mm diameter pipes. If the appropriate averages were 50% higher than the £20 and £35 per m I have used above, the marginal annuitized costs rise to 56 and 71 p per tonne CDE for renewing low- and medium-pressure mains.

The levels of fossil methane in the atmosphere appear to imply<sup>6</sup> up to 6–9% leakage of gas worldwide. In direct terms, the gas industry is a big contributor to UK CO<sub>2</sub> emissions. Burning 18,800 million therms gives 103 megatonnes CO<sub>2</sub> per yr, amounting to one-sixth of the UK total. But the avoidable leakage (908 million therms on the median figures) contributes to the global greenhouse almost as much in CO<sub>2</sub> equivalence, and possibly 2 or 3 times higher.

It seems from my calculations that this avoidable leakage can be saved at very low cost, and that accelerated replacement of service pipes could even save money for British Gas. Even if gas leakage were at the much lower level claimed by the company, so that pipe-replacements save only 15–20% of the amounts used in the table, the costs at less than £3 and probably less than £1 per tonne CDE are still relatively low. The present programme for replacing pre-1969 gas piping would take 45 yr for gas mains and 15 yr for services at current rates. The replacement of service pipes has been cut to only 2.1% per yr, below the 2.5% per yr that the King report<sup>7</sup> (on gas explosions) considered "reasonable". The case for accelerating this programme to reduce global greenhouse pollution is surely very strong. □

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GAS LEAKAGE RATES

|                                       | Low-pressure<br>(therms per<br>km per yr) | Medium-pressure<br>(therms per<br>km per yr) | Services<br>(therms per<br>yr) |
|---------------------------------------|-------------------------------------------|----------------------------------------------|--------------------------------|
| Basic leakage Joints<br>(median case) | 4,875                                     | 13,370                                       | 52.5                           |
| Breaks                                | 3,600                                     | 9,880                                        |                                |
| Reduced rate*                         | 4,684                                     | 6,162                                        |                                |
| New pipe leakage†                     | 177                                       | 353                                          | 2.0                            |
| Net savings                           | 4,507                                     | 5,809                                        | 50.5                           |

Rates are recalculated from data in ref. 1.

\* Adopting median-case figures<sup>1</sup>: conditioning 15% effective for low-pressure mains, 65% effective for medium-pressure mains, repair of breakages 85% effective.

† Partitioning the total leakage of post-1969 pipes assumed as 47 Mtherms per yr (0.25%) between medium-pressure mains, low-pressure mains and services, per km length in the ratio 2:1:1; lengths of medium- to low-pressure mains in the ratio 1:6.4.

factor of 2. This differs from the IPCC figures<sup>5</sup> of 21 and 63 derived for 'time-horizons' of 100 and 20 years because I have allowed for extra indirect atmospheric effects and assumed effective discount rates (of 1–2.5% per yr) instead of time-horizons. With the methane factor of 50, the marginal discounted annual costs of CDE savings then become 25, 40 and –3 p per tonne CDE, for accelerating the renewal of low- and medium-pressure mains and service pipes, respectively, at 10% discount rate. For a higher 'commercial' discount rate of 13%, the annuitized mains cost double and the service replacement cost is +3 rather than –3 p per tonne CDE.

Such costs are very low compared with the previous estimate of £25 per tonne CDE (ref. 4). They are also very low compared with typical values of £5–10 per tonne CDE for various greenhouse-gas abatement technologies. The total saving via pipe replacement on the figures in the table is 908 million therms per yr (4.8% of production) or 90 megatonnes CDE, 2–3 times that achievable via technologies such as energy-efficient lighting or building pressurized water

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# Gene therapy on the move

Unexpectedly rapid advances in basic science suggest that gene therapy for a whole host of diseases will soon be a medical reality. The ethical dimensions — real and imagined — need to be sorted out.

THE intriguing notion that one might be able to cure genetic disease by giving patients a functioning version of a defective gene has been under consideration for the better part of two decades. At the outset, single-gene defects were the imaginary targets of would-be gene therapists who envisioned inserting functional genes into pluripotent stem cells in the bone marrow in order to cure a list of diseases that included sickle cell anemia, the thalassemias, the severe combined immunodeficiency disorders, and Lesch-Nyhan syndrome.

But stem cells, few in number, have proved remarkably resistant to isolation and manipulation. Although recent studies suggest progress on the stem cell front, during the past few years investigators turned their hands to a host of alternative cellular vehicles for gene therapy.

Thus it was that lymphocytes (in this case tumour-infiltrating lymphocytes, or TIL) were enlisted for the first approved human gene experiment in the United States, which took place in 1989 at the National Institutes of Health (NIH), where Steven A. Rosenberg, W. French Anderson and R. Michael Blaese used TIL to carry a marker gene for Neomycin resistance into the tumours of patients in the terminal stages of melanoma. A year later at the NIH, the same trio conducted the first trial of actual gene therapy when they transfused a little girl with adenosine deaminase deficiency (ADA) — a lethal immune disorder — with lymphocytes bearing the ADA gene, courtesy of a retroviral vector.

The ADA experiment (the child is doing well) is gene therapy in what was envisioned to be the classic case. By inserting the appropriate gene, which expresses an intended protein, one corrects a single-gene disorder.

However, in just the two years that human gene trials have been underway at the NIH, it has become clear that gene therapy is not just for single-gene disorders any more. Last week at an NIH conference simply titled "Human Gene Therapy", a record crowd of more than 550 researchers — academic and corporate alike — came away convinced that one day in the not too distant future, gene therapy will become a potent new force in medicine, with application to diseases as diverse as heart disease, cancer in its many forms, liver disease and diabetes — among others.

Claude Lenfant, director of NIH's Na-

tional Heart, Lung and Blood institute (a co-sponsor of the conference), even predicted that gene therapy will eventually play a role in disease prevention, by correcting LDL receptor deficiency right after birth, for instance. And, of course, hope for the treatment of the major single-gene diseases such as cystic fibrosis (CF) was high. Francis Collins of Ann Arbor (co-discoverer of the CF gene with Tsui and Riordan of Toronto) put that in perspective, however, with this quote from the diary of an 8-year-old CF patient. Dated 25 August 1989, she wrote "Today is the best day ever in my life. They found a Jean for Cistikfibrosis." Of course, finding the gene — as Collins pointed out — has not benefited the patient at all as yet, but, he predicted, it will one day soon.

The cellular vehicles for potential gene therapy are multiplying with unexpected speed, now including not only lymphocytes but also epithelial and endothelial cells, and myoblasts. In addition, retroviruses are yielding their primacy as vectors for gene insertion to DNA viruses (adenovirus in particular) and synthetic vehicles such as Ligand-Polylysine-Minigen complexes.

The list of existing and pending gene therapy trials now stands in the US at six gene-transfer and five gene-therapy studies underway, with an additional seven likely to win approval early in 1992. One study has just been approved in the Netherlands. Many more are sure to follow.

All of this means that it is now particularly important to sort out the social/ethical/policy issues that come up whenever gene therapy is mentioned. One of them is the 'mutant' issue, best dealt with by a patient who, after receiving a transfusion of genetically modified lymphocytes, quipped that it must be safe because "I haven't turned into a mutant."

In terms of responding to public fears about gene therapy, fear of the mutant, while groundless, should nevertheless be at the top of the agenda, according to Georgetown University Law School dean Judith Areen, who reminded the NIH conference that the ordinary person confuses Dr. Frankenstein with his monster, and that the two have been "fused in the public mind" with scientists of all disciplines. Combine that unhappy fact of life with the horrors of eugenics as advocated by Nazi Germany and it is easy to see why Areen says that, like it or not, gene therapists have what lawyers call the 'burden of

proof' in this field of science and medicine."

Fears are widespread. Information is limited. All the more reason to make sure the public is privy to the decisions that are made. It is equally important that scientists discuss them clearly and in context. For instance, as Anderson acknowledges, the possibility that a retroviral vector will some day integrate next to a tumour suppressor and lead to cancer is real. "Sooner or later," he said, "some patient will get cancer [given the known imperfections of current vector technology]."

Should that inevitably put gene therapy on hold? Most would argue that it should not, presuming that the seriousness of the disease being treated and the absence of alternative therapies ethically justifies choosing risk in a risk-benefit analysis. What is important is that the public, to the extent that it cares to listen, be told exactly what the risks are — no more, no less. It is worth noting, here, that society already has ample experience in this area, even though it is seldom discussed openly in unambiguous language. Many chemotherapeutic drugs — drugs that have been used in cancer therapy for years — are themselves carcinogenic. Data show that patients who are cured on one cancer and live long enough are vulnerable to developing a new tumour attributable to the initial life-saving chemotherapy.

The point here is not that no ethical issue exists but rather that the ethical issue posed by the use of retroviral vectors in gene therapy is not entirely beyond existing medical experience and should be seen as part of a continuum.

Another so-called ethical issue is cost. Gene therapy trials, conducted for now in high-technology medical centers, are expensive. But cardiac surgery and transplantation therapy are surely equally expensive. If cost is an issue, it is not unique to gene therapy. Nor are questions about patient privacy, or reproductive rights, or the behaviour of insurance companies who want to exclude patients with genetic disease.

The discussion of ethical issues has, for the present, something of an abstract air about it. But that will not last. Once gene therapy is a real, practical part of medicine, the imaginary fears will probably evaporate, and the other issues will have to be faced just like any others in medicine.

Barbara J. Culliton



# Dwarf galaxies' vanishing act

Cedric Lacey

ASTRONOMERS try to learn how galaxies have evolved over time by observing objects at great distances from us, which we see as they were when the Universe was younger. Over the past decade, improved observational techniques have allowed the investigation of ever more distant samples of galaxies. Now Cowie *et al.*, on page 460 of this issue<sup>1</sup>, report measurements of redshifts (look-back times) for the faintest sample of field galaxies yet, which are seen as they were when the Universe was 30 per cent younger (perhaps by 3–4 billion years) than now. These observations seem to indicate that low-luminosity dwarf galaxies were much more numerous then than they are today. This result is very surprising, as it implies that the galaxy population has since evolved rapidly and, if true, will require a substantial revision of current ideas about galaxy evolution.

To study the evolution of typical galaxies (rather than exceptional objects such as radio galaxies or quasars), objects are detected through the visible or infrared light emitted by their stellar populations. Because galaxies seen at fainter fluxes are on average expected to be more distant, the first stage is simply to count the number of galaxies per unit area of the sky down to as faint a limiting flux as possible. These galaxy counts provided the first direct evidence of evolution of the normal field galaxy population, in that the number of galaxies seen per unit flux (or apparent magnitude) interval increases more steeply with decreasing flux than is expected for a nonevolving population, this excess reaching a factor of 5–10 for the faintest galaxies detected in the (blue) B-band<sup>2</sup>.

## Stellar evolution

However, the counts alone do not uniquely determine the form of the evolution. The first galaxy evolution models studied were in a sense the simplest ones possible, in that galaxies were assumed to evolve as isolated units, forming at high redshift, turning their gas into stars on timescales that depended on their morphological type (spiral or elliptical) but not their mass, and changing luminosity as their stars evolved. These pure luminosity evolution models<sup>3,4</sup> predicted that typical galaxies should have been significantly brighter in visible bands in the past than now, allowing them to be seen out to larger distances (or redshifts) than otherwise, sampling a larger volume and thus providing a natural explanation for the excess in the number

counts.

The crucial prediction of these luminosity evolution models — that the redshifts of galaxies seen at a given flux should be larger than for a no-evolution model — has become testable only recently, because measuring redshifts of faint galaxies is more difficult than measuring fluxes. The initial results of such measurements<sup>5,6</sup> were surprising, as the mean redshifts found did not differ significantly from the no-evolution prediction, in spite of the evidence for evolution from the increased number counts at the same flux. This finding has now been extended to a new fainter flux limit by Cowie *et al.*<sup>1</sup>, who measure a median redshift of 0.4 in the magnitude (brightness) interval  $23 < B < 24$ , where the excess in the counts is a factor of 3–5. The implication is that one of the assumptions of the simple luminosity evolution models — that the number of galaxies is conserved or that galaxy evolution is independent of mass — is wrong.

## Young stars

Important additional information on galaxy evolution is provided by the recent advent of observations of faint galaxies in the near-infrared K-band<sup>7</sup>. For the redshifts of interest ( $z \geq 0.2$ ), the light in the B-band is dominated by young stars, so the measured fluxes are affected strongly by galaxy evolution, and mainly trace the rate of star formation. On the other hand, the K-band light is produced by stars of all ages, so the measured fluxes are fairly insensitive to evolution, and mainly trace the total mass in stars. K-band luminosities therefore provide a convenient, fairly model-independent, means of comparing galaxies at different redshifts.

Cowie *et al.* combine their K-band flux and redshift measurements to make some inferences about the galaxy population producing the excess counts. They find that around half of the objects seen at  $23 < B < 24$  are dwarf galaxies at redshifts  $z \approx 0.3$ , which are 10–100 times less luminous in the K-band, and 10–100 times more numerous per unit volume, than typical bright galaxies (with luminosities of about  $10^{11}$  suns) found nearby. The remainder, at rather higher redshifts, have K-band luminosities comparable to nearby bright galaxies. In addition, the dwarf galaxies are quite blue, suggesting that most of their stars are fairly young compared with the more luminous galaxies, whose redder colours are consistent with star formation at high redshift ( $z > 1$ ). The puzzle

is that, at the present day, the number density of such dwarfs is thought to be about 10 times smaller than this, so where have they all gone?

There are several possibilities, but all have problems. First, if the dwarfs have ceased star formation, they would by now have faded significantly in the B-band, in which local galaxy counts have been performed. But it seems unlikely that they could have faded enough to evade detection unless the initial distribution of stellar masses in these galaxies was deficient in the roughly solar-mass stars that would produce most of the light today. A direct determination of the local luminosity distribution in the K-band would be extremely valuable to check for the existence of such a population of faded galaxies, as would an accurate determination of the number density of low surface brightness galaxies in the field.

## Self-destruction?

The second solution is that the dwarfs might have self-destructed as a result of supernova explosions of young stars blowing away most of their gas, causing the galaxy to become gravitationally unbound. However, typical luminous galaxies are known to be surrounded by halos of dark matter containing 10 times as much mass as there is in visible form; such halos around dwarf galaxies, if present, would gravitationally confine the stars, preventing disruption, even if all the gas were ejected.

Lastly, the dwarf galaxies might have disappeared by merging into more luminous galaxies. This comes up against the problem that the total stellar mass in the dwarfs is required to be comparable to that in brighter galaxies, according to the authors' estimates (again assuming a normal distribution of stellar masses), so that such merging would significantly affect the properties of the present-day population of bright galaxies into which the dwarfs are incorporated. The thinness of the disks of modern spiral galaxies implies that they cannot have accreted more than about a tenth of their mass in recent mergers with other galaxies<sup>8</sup>, and the red colours of elliptical galaxies imply that most of their stars formed long before those seen in the dwarfs.

Perhaps theorists will have to give up

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their convenient assumption that stars are everywhere born with the same distribution of masses, which they have been loath to do because of the enormous additional uncertainty this would introduce into the models. A more precise determination of the number of dwarf galaxies is obviously crucial — Cowie and co-workers' redshift sample

## PLANETARY ASTRONOMY

## Icy clues to Triton's origins

William B. McKinnon

FROZEN carbon monoxide and dioxide have been discovered on Triton, Neptune's largest moon. D. P. Cruickshank (NASA Ames) and colleagues reported at a meeting last month on planetary sciences\*. Not only is this the first ever detection of these ices on a moon or planet in the Solar System, but it may bear on the question of whether Triton once followed its own independent orbit about the Sun, only to be captured by its giant partner.

The measurements were made last July at the 3.9-metre United Kingdom Infrared Telescope (UKIRT) on Mauna Kea, Hawaii, using a new spectrometer that is 16,000 times more sensitive than previous detectors. Greatly increased spectral resolution allowed detection of narrow infrared features that were previously considered to be almost impossible to discriminate against the dominant and complex methane absorption bands that Triton presents. Cruickshank and colleagues detected multiple spectral bands for both CO and CO<sub>2</sub> ice, and on more than one night, and the band positions were confirmed with laboratory transmission spectra of solid CO and CO<sub>2</sub> at temperatures comparable to that of Triton's surface (38 K).

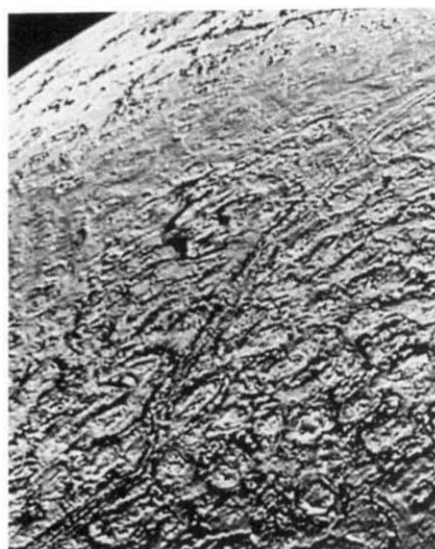
These detections are important primarily because they may represent direct cosmochemical proof that Triton was originally captured from solar orbit. CO especially has had the status of a grail species, because it is abundant in star-forming molecular clouds, was ostensibly abundant in the equivalent solar nebula from which the Solar System emerged, and is found (at the few-per-cent level) in comets such as Halley<sup>1,2</sup>. It may also be the dominant heavy gas in Pluto's atmosphere required to explain the planet's June 1988 stellar occultation curve<sup>3</sup>. But CO is not supposed to have been a major constituent of the satellite-building nebulae that once existed around the giant planets, so its detection on Triton (already thought to be Pluto's near twin<sup>4</sup>) is at least consistent with the idea that the satellite has a comet-like

contains only 20 galaxies. But in any case, it seems clear that interesting evolution in the galaxy population has occurred quite recently, contrary to initial expectations. □

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composition.

But in finding CO, have Cruickshank and colleagues really found the 'sublimating gun' revealing Triton's origin? Possibly not. Although further detailed analysis is necessary, Cruickshank and co-workers estimate that both CO and CO<sub>2</sub> are only trace components in the surface ice, which is mainly solid nitrogen with a relatively small amount (ab-



A permanent visitor? A Voyager 2 image of Triton, looking across the so-called cantaloupe terrain towards the southern polar cap, which incompletely buries the surface topography. The quasi-circular features at the bottom have a characteristic scale of about 40 km.

out 1 per cent) of methane ice<sup>5</sup>. At the trace level, CO could be photochemically derived from CO<sub>2</sub>, which may be outgassed from Triton's interior, whatever its origin. And it may be unreasonable to expect much primordial CO at Triton's surface, for Triton may have gone through a protracted episode of extreme heating owing to tidal effects following its capture by Neptune<sup>6,7</sup>. In this case, hydrothermal processing of molten cometary ices or dissolved CHON organics, at the top of Triton's hot rock core, would have largely converted any CO into CO<sub>2</sub> or organic material, depending on the oxidation

state of the hydrothermal system<sup>8,9</sup>. In addition, materials as volatile as CO would have formed an atmosphere when Triton's surface temperature was driven up by the heating caused by its tidal interaction with Neptune, and could have been lost through hydrodynamic escape.

Almost incidentally, the new spectral measurements confirm the nitrogen absorption feature detected by Cruickshank and Apt<sup>10</sup> at a wavelength of 2.15  $\mu$ m. It was the strength of this feature that prompted the widely discussed hypothesis that Triton has oceans made up of liquid nitrogen. Nowadays, it seems safer to suggest that a deep, clear layer of N<sub>2</sub> ice is responsible, as the transparency of solid N<sub>2</sub> rivals that of water ice, and surface nitrogen deposits on Triton should rapidly anneal, on a seasonal timescale<sup>11</sup>. It is also interesting to note that water ice, which is almost certainly the principal ice on Triton and which is inferred to provide the strength necessary to support the satellite's surface topography, cannot be seen in the new high-quality spectra. Apparently it is overwhelmed by optically active methane ice.

What remains to be done is to carefully evaluate the full spectrum, determining what other chemical species may be trapped in Triton's ices. These can then be matched against known cometary volatiles and their plausible photochemical and thermochemical descendants. Pluto will also be observed and its surface CO/N<sub>2</sub> ratio determined, and this will be a key clue to how Pluto's evolution differed from Triton's. Given the power of the new spectrometer, we should look forward to many important discoveries, such as the identification of minor species associated with geologically recent volcanism on Jupiter's Europa and Uranus's Ariel, and perhaps even a fix on what makes up the dark side of Saturn's moon Iapetus. □

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\*Division for Planetary Sciences of the American Astronomical Society, Palo Alto, 4–8 November 1991.

# In the beginning . . .

Dennis J. Selkoe

ADDED momentum to the molecular assault on the underlying cause of Alzheimer's disease will stem from the paper by Kawabata *et al.* that appears on page 476 of this issue<sup>1</sup>. The authors describe the most useful animal model yet for the disease — a mouse expressing a 100-residue C-terminal fragment of  $\beta$ -amyloid precursor protein which develops several of the features seen in the brains of Alzheimer's patients.

Despite doubts expressed over the years, studies of the origin and effects of cerebral amyloid deposition continue to provide clues to early events in Alzheimer's disease. Circumstantial evidence for the central involvement of the amyloid  $\beta$ /A4 protein, which consists of about 40 residues, has long been building, but this year has witnessed two developments which provide 'smoking guns' for the amyloid hypothesis. First, missense mutations at codon 717 of the gene encoding the 770-residue  $\beta$ -amyloid precursor protein ( $\beta$ -APP) have been found in affected members of a handful of families with autosomal dominant Alzheimer's disease<sup>2-6</sup>. Second, studies of transgenic mice overexpressing some or all of the human  $\beta$ -APP molecule have started to bear fruit<sup>7,8</sup>. The latest entry into the parade of mice looks to be the most intriguing yet.

During ageing, humans, monkeys and certain other mammals develop extracellular deposits of  $\beta$ /A4 protein, called 'preamyloid' or 'diffuse' plaques, which are largely nonfilamentous and lack the dystrophic neurites (axons and dendrites) that surround the filamentous amyloid cores of so-called 'mature' plaques. In Alzheimer's disease, a much greater number of diffuse  $\beta$ /A4 deposits occur, and many of them seem to have progressed to a compacted, filamentous stage associated with structurally altered neurites. This heightened formation of mature  $\beta$ /A4 plaques is usually accompanied by myriad neurofibrillary tangles, bundles of paired helical filaments in the neuronal cytoplasm that are principally composed of the microtubule-associated protein, tau. Before now, such neurofibrillary tangles had not been observed in mammals lower than man. In humans with trisomy 21 (Down's syndrome), diffuse  $\beta$ /A4 plaques may begin to accumulate in the teens or twenties and herald the development of the full-blown neuropathology of Alzheimer's disease by the late forties.

Until this year, the only compelling animal model of Alzheimer brain pathology was the aged monkey<sup>9</sup>. But there are severe constraints on studying pri-

mates, and a mouse expressing full-length human  $\beta$ -APP751 driven by the neuron-specific enolase promoter provided the first manipulable model<sup>7</sup>. These animals displayed extracellular deposits in hippocampus and neocortex that reacted with monoclonal antibodies to synthetic  $\beta$ /A4, but no surrounding neuritic dystrophy, neurofibrillary tangles or other Alzheimer-like pathology has yet been described in them.

The mice now reported by Kawabata *et al.*<sup>1</sup> express just the  $\beta$ /A4 region plus the cytoplasmic tail of human  $\beta$ -APP; transcription is driven by the neuronal-specific Thy-1 promoter. This C-terminal fragment was chosen because earlier studies suggested it could self aggregate *in vitro* to make polymers<sup>10,11</sup>, and that it may be toxic when transfected into mammalian cells<sup>12</sup>. By four months of age, two founder lines exhibited  $\beta$ /A4-immunoreactive deposits resembling diffuse plaques. By eight months, one line had developed numerous  $\beta$ /A4- and silver-positive plaques in hippocampus, amygdala and neocortex, including some with compacted cores and some associated with altered neurites. Moreover, there were neurofibrillary tangles that reacted with silver, thioflavin S (a classical amyloid-binding dye) and the tau monoclonal antibody, Alz-50, and thus closely resembled Alzheimer tangles. Reductions in the number of pyramidal neurons in hippocampus and Purkinje cells in cerebellum were seen.

Taken together with the earlier observations<sup>7</sup>, this striking neuropathology leads to several conclusions. First, high neuronal expression of  $\beta$ -APP, in particular the  $\beta$ /A4-containing C terminus, can lead to extracellular  $\beta$ /A4 deposits followed by the development of neuritic plaques and neurofibrillary tangles. This fits well with the temporal progression of these lesions in humans with Down's syndrome, in whom structurally normal  $\beta$ -APP is overexpressed. The long-standing argument over the primacy of plaques and tangles in Alzheimer's disease has been settled in favour of plaques (although tangles and dystrophic neurites are undoubtedly clinically important). The results from transgenic mice, coupled with the discovery in some Alzheimer families of an amino-acid substitution in the transmembrane domain of  $\beta$ -APP near  $\beta$ /A4, indicate that amyloidogenic processing of  $\beta$ -APP can precede all other manifestations of Alzheimer's disease and thus be truly causative of the disorder, although probably by way of a slow, multi-step degenerative cascade<sup>13</sup>. But it remains likely

that other molecular mechanisms, such as changes in proteases, kinases or other enzymes involved in the correct post-translational processing of  $\beta$ -APP, can also initiate  $\beta$ -amyloidosis in some Alzheimer patients.

Second, the expression of a construct beginning with the methionine-aspartic acid bond at position one of  $\beta$ /A4 leads to surprisingly rapid development of extracellular  $\beta$ /A4 deposits in young mice, signifying that cleavage at or near this bond is probably a requirement for producing amyloidogenic  $\beta$ -APP fragments and the release of the fragments into the neuropil. The enzymes that cleave this bond and at the C terminus of  $\beta$ /A4 are worthy targets for inhibition in order to reduce  $\beta$ -amyloidosis.

Finally, the high expression of a  $\beta$ -APP fragment in neurons throughout the brain can lead to the preferential formation of amyloid plaques in the hippocampus, amygdala and neocortex. This result supports the notion that emerges from the histopathology of the human disease: that unknown factors in these brain regions promote the development of mature plaques bearing filamentous amyloid and surrounded by structurally altered axons and dendrites. In Alzheimer's disease, clinical symptoms reflect the topography and density of neuronal, neuritic and glial changes, not the distribution of  $\beta$ /A4 deposits as such. The mechanism whereby an apparently aggregated, fibrillar form of  $\beta$ /A4 exerts its effects on cortical cells — whether directly<sup>14</sup>, indirectly<sup>15</sup> or as a matrix that binds various biologically active  $\beta$ /A4-associated proteins<sup>13</sup> — remains unknown.

As exciting as the neuropathology of the new transgenic mouse appears to be, the model has its limitations. The construct being expressed is not known to occur *in vivo*, and the sequential proteolytic cleavages of full-length, glycosylated  $\beta$ -APP that lead to amyloidogenic fragments, and ultimately to extracellular  $\beta$ /A4, are likely to differ from the processing of the transgene product. In this regard, Kawabata *et al.* emphasize that it is not yet clear whether the transgenic deposits included the  $\beta$ -APP C terminus or only the  $\beta$ /A4 peptide itself. There is currently no evidence that diffuse and mature extracellular  $\beta$ /A4 deposits in humans contain the C terminus or other non-amyloid regions of the precursor, although a subset of mature plaques contains dystrophic neurites that react with antibodies to the N- and C-terminal domains of  $\beta$ -APP<sup>16</sup>. Importantly, the endogenous  $\beta$ -APP gene is expressed in virtually every cell type in both neural and non-neural tissues, in contrast to the exquisite neuronal specificity of the Thy-1 promoter. This difference may account for the lack of



microvascular amyloidosis characteristic of Alzheimer's disease. The striking neuronal loss observed in the mouse includes cell types (Purkinje cells, for example) that are not generally affected in Alzheimer patients, and the neuronal degeneration is not always associated with the local presence of plaques and tangles. Finally, much of the cell death in the mouse may stem from neurotoxicity from very high intracellular expression of the transgene, and the production of an Alzheimer-like phenotype has been compressed into a remarkably short time, contrasting with the indolent course of the human disease.

Over the past year, the outlines of further experiments have become clear. Examining the detailed temporal progression of  $\beta$ A4 deposition and the ensuing biochemical and morphological alterations in the mice of Kawabata *et al.* should help clarify which  $\beta$ A4-associated molecules and which reactive cells are involved in the formation of mature neuritic plaques. Immunocytochemistry at the light and electron microscopic levels, coupled with *in situ* hybridization, should help establish precisely which neuronal proteins are expressed differently or accumulate during the early stages of fibrillary degeneration of neurons and their processes. Of special importance will be the characterization of transgenic mice expressing  $\beta$ -APP molecules with the mutations at residues 693 or 717 described in humans<sup>2-6,17</sup>.

Increasing attention will also centre on the alternative proteolytic pathway in cells that generates  $\beta$ A4-containing fragments, the part of lysosomal proteases in this process, and the mechanism of release of the fragments into the extracellular space, where they are further processed into amyloid filaments. The search for other mutations in coding or regulatory regions of  $\beta$ -APP may ultimately yield an array of defects in this gene analogous to the 20 or so mutations identified to date in the transthyretin protein that underlie various forms of

familial amyloidotic polyneuropathy<sup>18</sup>. From these and other studies, strategies to modify amyloid production or toxicity should emerge. Promising compounds can be screened in cells overexpressing  $\beta$ -APP and in transgenic mice.

Finally, it should be borne in mind that the distinction between Alzheimer's disease and ageing of the brain is essentially quantitative rather than qualitative. So elucidation of the genesis of HIV

plaques and tangles in Alzheimer's disease may well tell us a great deal about the similar lesions that in part underlie the subtle changes in memory and cognition affecting some otherwise healthy septuagenarians. □

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## Games that viruses play

Rolf M. Zinkernagel and Hans Hengartner

VIRUSES use many tricks to persist in an immunocompetent host, one of them being mutation of the sequences (epitopes) recognized by the host's viral-specific cytotoxic T cells. Are such T-cell epitope escape mutants to be found in HIV-positive patients? Earlier this year, Meyerhans and colleagues<sup>1</sup> reported that they were not. But in their paper on page 453 of this issue<sup>2</sup>, Phillips *et al.* now describe the identification of such HIV *gag* T-cell epitope mutants. The new result has a precedent in studies of mice infected with lymphocytic choriomeningitis virus, in which it was demonstrated, *in vivo*<sup>3</sup> and *in vitro*<sup>4</sup>, that the antiviral cytotoxic T-cell response may select viruses expressing an altered epitope that allows them to escape immune surveillance.

Phillips *et al.* isolated HIV repeatedly from patients over two to four years and tested whether the HIV-specific cytotoxic T-cell responses of the same patients were able to recognize the HIV isolates. They found a few examples in which an altered T-cell epitope was expressed, so that the new variant was not recognized by the T cells specific for the original epitope. Whereas in some examples the new epitope was not recognized by the patients' newly induced T cells, another epitope variation was still recognized. So the variant viruses had apparently not (yet?) escaped immune surveillance entirely.

Because antiviral CD8<sup>+</sup> T cells can recognize antigen only as small peptides presented by class I MHC antigens<sup>5</sup>, which vary widely in a given population, such selection is probably more important within an individual than in a population. But because viruses seem to exhibit only a few T-cell epitopes recognized by CD8<sup>+</sup> T cells it may be that selection occurs in several steps, eventually reaching all epitopes. If this were to occur over several rounds of selection through several individuals in sequence, viruses could result that exhibit weak or no T-cell epitopes.

There are several limitations to the

observations and to their interpretation. First, as Meyerhans *et al.* and Phillips *et al.* point out, HIV immunology is difficult because one can usually work only with blood lymphocytes (HIV from lymphoid tissue may differ) and restimulation of CD8<sup>+</sup> T cells with the patient's own cells may not reveal all the activity present. Second, firm identification of antivirally protective CD8<sup>+</sup> T cells is not really possible yet, so, for HIV, any conclusions must remain tentative. Finally, only a few patients have yet been analysed. In those studied so far, not all HIV isolates exhibited altered T-cell epitope(s). Also several HIV variants obviously coexist (which probably explains why in the study of Meyerhans *et al.*, in which HIV epitope presented by HLA B27 in four patients was monitored, no variant virus was found). Finally, neither the balance between the mutants themselves, nor between the variants and the T-cell response, is known. So the results of Phillips *et al.* are not decisive. But they are compatible with the notion that T-cell epitope escape mutants may play some role in AIDS.

The question is, what might that role be? Together with earlier work (reviewed in ref. 6) the findings suggest that, as is known for other virus infections, CD8<sup>+</sup> T effector cells are involved in controlling the spread of HIV within an individual. All these studies therefore imply that the CD8<sup>+</sup> cells help to protect the patient, but that is not necessarily always the case; the same CD8<sup>+</sup> T cells also cause destruction of infected cells (that is, immunopathology). In spite of all we have learned about HIV, it is still not known for sure whether it destroys the cells in which it replicates in the course of a natural infection. If, as we think likely, HIV is noncytolytic in infected hosts, the immunodeficiency it provokes may be both caused and prevented by the cytolytic T-cell response; which becomes more visible depends on the balance between the spread of the virus and the immune response<sup>7</sup>. ►

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In the infection of mice with lymphocytic choriomeningitis virus (LCMV), which is also an RNA virus, but not a retrovirus, and which causes a much more acute form of immunosuppression, we found evidence for such a pathogenesis of immunodeficiency. Like HIV, at the beginning of the course of infection LCMV predominantly infects macrophages, dendritic cells and antigen-presenting cells in general. If too many of these cells are infected, then their destruction by the CD8<sup>+</sup> T cells prevents further immune response<sup>7,8</sup>.

How could the possibility that AIDS results from T-cell mediated immunopathology be supported? First, by the observation that HIV infection of the embryo before 7–10 weeks of gestation (that is, before T cells have become immunocompetent) might cause an asymptomatic carrier infection associated with tolerance to the virus. And second, there is of course the selection of T-cell epitope escape mutants, as described by Phillips *et al.* By their very existence (or at least persistence), true T-cell escape mutants, which were no longer recognized by any of the cytolytic T cells in a patient's polyclonal immune response, would support the idea of a T-cell mediated disease. Were HIV a cytopathic virus, the 'playground' for the mutation game would be limited, and successful escape from the immune response would accelerate disease and thereby impair the survival of the virus. By contrast, if HIV causes cell destruction by T-cell mediated immunopathology, such escape mutants persist and should cause less or no disease.

Perhaps, therefore, the consequences of such an HIV virus, able to escape recognition by cytotoxic T cells at the population level, need not be too worrying. With its ability to avoid clearance by the immune system of the host, its ability to exploit immunological stimulation to infect new cells, and its ability to replicate in an astonishingly balanced fashion over many years, HIV has already demonstrated that it can play immunological games to an extent and with a finesse that reveal the very biological limits of the immune system. □

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## A cytoplasmic chaperonin?

Geoffrey North

SEQUENCE similarities often spring surprises. A particularly striking example is the discovery, reported at a recent European Research conference\*, and in part on page 490 of this issue<sup>1</sup>, that a protein product of a thermophilic archaeobacterium is closely related to a protein encoded by a mouse t-complex gene. The upshot may be the identification of a cytoplasmic chaperonin, a class of molecular chaperone that is thought to play a central part in protein folding *in vivo*.

Molecular chaperones are cellular components that are believed to assist the folding, translocation and assembly of proteins in cells, preventing inappropriate interactions from occurring in the complex, highly concentrated cellular environment (a review of the topic will shortly appear in these pages<sup>2</sup>). Several classes of them are known, defined primarily by their molecular weight and sequence. Of particular interest are the chaperonins, a class whose members are variously named according to their different origins. But they always consist of a protein of relative molecular mass 60,000 ( $M_r$  60K) with a highly conserved sequence; in some (but not all) cases, the 60K protein is known to have a 10K partner — hence the terms chaperonin 60 (cpn60) and chaperonin 10 (cpn10)<sup>3</sup>.

In bacterial chaperonins, the two types of subunit assemble into oligomeric complexes. Bacterial cpn60, groEL, forms a complex of two face-to-face seven-membered rings; a further seven-membered ring of bacterial cpn10, groES, interacts with one face of the groEL complex. Such complexes seem to form sites on which nascent proteins can fold, and on which they are protected from interactions that would otherwise lead to aggregation<sup>4,5</sup>.

Although chaperonins are known from eubacteria and organelles (where they are often referred to as hsp60s), a cytoplasmic chaperonin has been conspicuous by its absence. That has been something of a worry for those who think that chaperonin complexes are of fundamental importance in protein folding.

The first hint of the existence of a cytoplasmic chaperonin came from R. J. Ellis<sup>6</sup> and R. S. Gupta<sup>7</sup>, who independently noted weak but apparently significant sequence similarity between chaperonins and a protein known as t-complex polypeptide 1 (TCP-1). The protein was first identified as the product

of one of the genes that make up the mouse t-complex, a collection of linked chromosomal rearrangements which are prevalent in wild mouse populations, and which are maintained by a combination of suppressed chromosomal rearrangement and segregation distortion. (Segregation distortion ensures that mice heterozygous for t and wild-type chromosomes pass on t-chromosomes to a high proportion of their progeny, despite the homozygous lethality of the t-complex.)

Given that the chaperonins form a highly conserved sequence class, the significance of the weak similarity in sequence was not clear. Now, however, support for the idea that TCP-1 is a chaperonin comes from an unexpected quarter. A. L. Horwich (Yale School of Medicine) has found that an abundant protein of the thermophilic bacterium *Sulfolobus shibatae*, known as TF55 (for thermophilic factor 55), is very similar in sequence to TCP-1 — so similar, indeed, that they are likely to have similar functions.

*Sulfolobus shibatae* lives at a preferred temperature of 75 °C, but it can acquire tolerance to 85 °C. This process is accompanied by the induction of further expression of TF55, which thus qualifies as a heat-shock protein. A number of heat-shock proteins have turned out to be molecular chaperones, and from the initial biochemical studies it seems that TF55 will be no exception. Thus TF55 has ATPase activity, and will bind unfolded proteins *in vitro*<sup>1</sup>. Most strikingly, it assembles into a complex remarkably like the groEL 'double-doughnut'. The TF55 complex similarly consists of two face-to-face rings, but they each consist of nine (occasionally eight) subunits rather than seven.

The connection between TF55 and TCP-1 is strengthened by the discovery that TCP-1 also assembles into a double-doughnut complex, and like TF55 the TCP-1 complex comprises two nine-membered rings (K. Willison, Institute of Cancer Research, London). The TF55 and TCP-1 complexes differ, however, in that the latter are apparently heterooligomeric, with at least seven types of subunit (so far classified only by their molecular weights).

Although it was originally discovered

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\*Biology of Molecular Chaperones, University of Kent, Canterbury, 28 September–3 October 1991.

as a product of a t-complex gene, TCP-1 is a conserved, generally expressed cytoplasmic protein. If it turns out that the TCP-1 complexes really have a function similar to that of the cpn60/cpn10 complexes, then they could be the sites on which most or all cytoplasmic proteins fold. Some preliminary evidence in support of this conjecture was reported by Horwich and E. Craig (University of Wisconsin-Madison), both of whom have found that conditional mutations of the yeast TCP-1 gene at the restrictive

temperature prevent synthesis of the active forms of cytoplasmic reporter proteins.

The new results also bear upon a taxonomic issue. They strongly reinforce the phylogenetic grouping of the nucleus and cytoplasm of eukaryotic cells with archaeobacteria, with mitochondria and chloroplasts separately grouping with eubacteria. □

Geoffrey North is deputy biology editor of Nature.

## OPTICS

# Photonic insulators

J. B. Pendry

SEMICONDUCTORS and insulators are special entities: their internal atomic arrangements diffract the valence electrons into closed bands and prevent them from conducting electricity. This gives the electronic engineer a clean slate on which to design his transistor by selectively introducing doping atoms into the structure, and there follow some of the electronic wonders of our age. The vital ingredients of this trick are the electron's wave-like nature and the arrangement of atoms with a periodicity comparable to the electron wavelength.

It is natural to ask whether a similar trick can be worked with another wave-like disturbance — light. In late October (*Phys. Rev. Lett.* **67**, 2295–2298; 1991) E. Yablonovitch, T. J. Gmitter and K. M. Leung claimed to have found a periodic dielectric structure within which photons in certain frequency ranges are completely forbidden. The interior of such a material would be insulated from the influence of these frequencies of electromagnetic wave. This week, Yablonovitch and colleagues show how the

photonic insulator can be doped, like a conventional semiconductor, to give impurity levels (*Phys. Rev. Lett.* **67**, 3380–3383; 1991).

Yablonovitch and his collaborators had two questions to answer: one for the theorist, the other for the experimentalist. First they had to devise a suitable dielectric structure with the photonic insulating property. Second, they had to address the question of whether that structure could be fabricated. Theorists have been working furiously on calculations which include the full vector nature of the photon field and can now make predictions about which structures will be insulators. Figure 1 shows the structure proposed.

A scaling property of the theory can be exploited to

make an experimental test: if a dielectric structure is a photonic insulator to photons of wavelength  $\lambda$ , then a structure doubled in size will be a photonic insulator at  $2\lambda$ . Yablonovitch exploited this result to test proposed structures by scaling them into the microwave region, with lattice parameters of 8 mm, and using conventional machining techniques to fabricate them. These giant photonic insulators were then probed with microwave radiation and the results, revealing forbidden frequencies at 13–16 GHz, confirm the predictions of theory. A much more severe challenge is to manufacture the

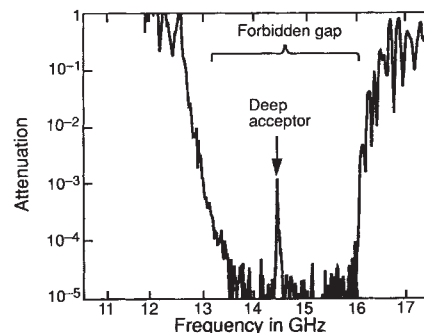


FIG. 2 Transmission in the  $\langle 111 \rangle$  direction through 8–10 layers of a photonic crystal. The gap falls between 13 and 16 GHz. A single acceptor, formed by removing dielectric material, is placed in the centre of the sample producing a deep acceptor state in the gap. (*Phys. Rev. Lett.* **67**, 3380; 1991.)

structure on a scale that would give a photonic insulator for visible radiation. Yablonovitch proposes that reactive ion etching could in principle make the required structure, albeit limited to a depth of around 20  $\mu\text{m}$ .



FIG. 3 Opals may be the closest natural equivalent to a photonic material, gaining their brilliant colours from diffraction effects.

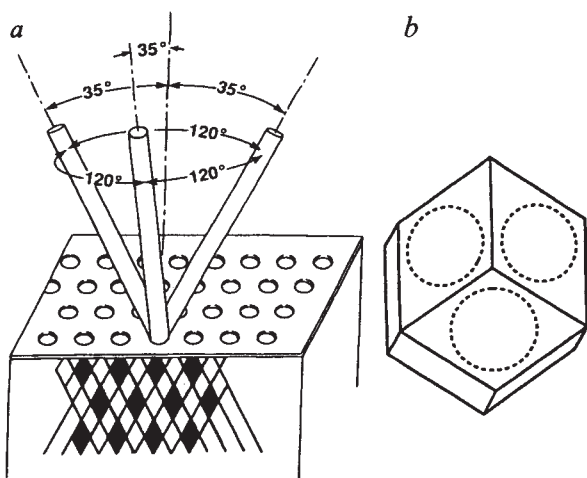


FIG. 1 *a*, The method used to construct a photonic insulator: a face-centred-cubic lattice of holes is drilled in a dielectric medium,  $\epsilon = 3.6$ . The drill enters each hole three times at three different angles. *b*, The unit cell of the structure. (*Phys. Rev. Lett.* **67**, 2295; 1991.)

What is so special about this material? Take an atom in an excited state that wants to decay back to the ground state by emitting a photon. Place the atom inside a photonic insulator and it cannot do so: each time it tries to escape, the photon is returned to the atom. Of course the excited atom does not live for ever because there will always be another decay path, but it could well live for a very long time, certainly much longer than it would in a normal material with a plentiful supply of photon states. This ability to tinker with something previously thought to be fairly immutable gives great scope for the design of lasers where lifetimes of excited species are crucial to obtaining population inversion. Thus in semiconductor laser structures, instead of being able to adjust only the electron bands to our convenience, the photon states can be designed to optimize performance. There is a



happy marriage of length scales: atomic scale modulations that affect electrons are on much too fine a scale to be noticed by photons, whereas the modulations in structure that diffract photons are on too large a scale to influence the electrons.

These materials would make excellent mirrors. Currently we rely either on metals, which have the disadvantage that they always absorb some of the light, or on total internal reflection from dielectric surfaces. The latter can have very low absorption but are only completely reflecting over a range of angles: the slightest surface imperfection allows light to escape. In contrast, a closed cavity inside a photonic insulator does not lose light by scattering. The only escape for the light would be absorption. So cavities of very small dimensions but of phenomenally high quality factor ( $Q$ ) could be built and within them resonant states could be pumped to high energy density. For example, if we could make a photonic insulator of the same quality as optical fibre in which light can travel for tens of kilometres without loss, then a cavity with dimensions of the order of the wavelength of light would have a  $Q$  of  $10^{10}$ , but still have excellent separation of the modes.

The cavity modes play the role of dopant levels in a semiconductor. Figure 2 shows the transmission coefficient of a structure fabricated with a cavity deliberately introduced into the structure. It is almost impossible to transmit electromagnetic waves through the sample in the forbidden region, except at the resonance frequency introduced by the cavity dopant. Here light tunnels into the cavity, and then out on the far side of the specimen. This is analogous to the resonant tunnelling mechanism by which electrons are transported across disordered semiconductors. The width of the peak gives the  $Q$  of the cavity, found to be  $10^3$  in this instance due to losses in the dielectric.

One of the more intriguing aspects of this new type of material is how it would appear to the eye. Certainly it can be expected to have gem-like qualities. The most similar gem stone we have available today is opal, which comprises a periodic array of tiny silica spheres. It is not a photonic insulator, but its rich colours (Fig. 3) are due entirely to diffraction phenomena. A perfect specimen of a photonic insulator, exhibiting strong dispersion, and having a huge effective refractive index over a range of frequencies, would be an object of great beauty. Like diamond, it would be both of use and an ornament. □

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## Crystal effects in $\beta$ -decay

J. J. Rehr

SOLID-STATE physicists and molecular biologists have become adept at interpreting minor details in X-ray absorption spectra to identify subtleties of crystal and molecular structure. On page 468 of this issue<sup>1</sup>, Steven Koonin points out that chemical environment could have a similar — but hitherto unnoticed — effect on the  $\beta$ -decay spectra of radioactive atoms.

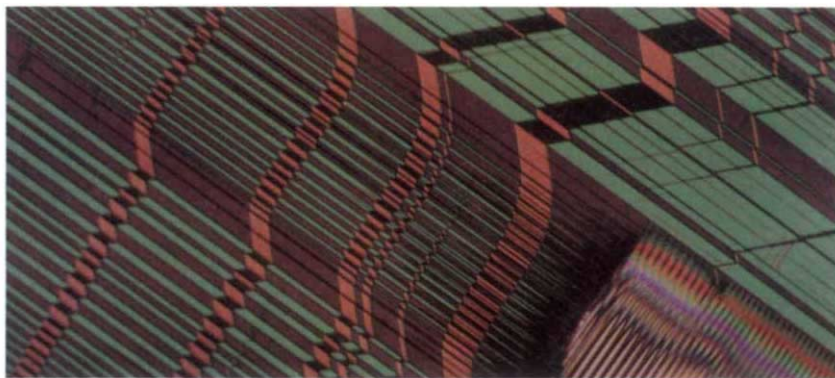
Naturally radioactive atoms often decay by emitting a  $\beta$ -particle (an electron or positron) in a fundamental process which transmutes the atom into the next element in the periodic table. The spectrum of  $\beta$ -decay energies led to the discovery of the neutrino in the 1930s: whereas  $\alpha$ -particles and  $\gamma$ -rays from radioactive decays come with characteristic single energies,  $\beta$ -decay energies reveal a broad spectrum. Fermi concluded that the radioactive energy was shared by the  $\beta$ -particle and an invisible neutrino.

More recently, the precise form of the spectra for the  $\beta$ -decay of a couple of nuclides has led to speculation about the existence of an unknown 'heavy' neutrino<sup>2</sup>. That issue remains hotly debated, because a number of mechanisms, the coulomb potential of the transmuted element for example, influence the shape of the spectrum. Koonin's new effect, the imposition of oscillations on the spectrum, which he names BEFS ( $\beta$  environmental fine structure) to echo EXAFS (extended X-ray absorption fine structure), needs to be added to this list.

The theory of BEFS is akin to the short-range-order theory of EXAFS. That idea was originally suggested by Kronig<sup>3</sup> in 1932. Kronig also developed an alternative, long-range-order theory, and it wasn't until the early 1970s and

the advent of continuously tunable synchrotron radiation X-ray sources, that the short-range-order theory was confirmed. According to this theory, the EXAFS oscillations seen in X-ray absorption spectra arise because of quantum mechanical interference between outgoing and reflected photoelectron waves when the de Broglie wavelength of electrons ejected by X-rays is comparable to the interatomic spacing in the material. Kronig's argument also accounts for the ubiquity of EXAFS-like oscillations. They have also been observed, for example, in photoemission intensities, Auger-electron spectroscopy, and near the Bragg peaks of X-ray diffraction patterns.

The oscillations that Koonin predicts in  $\beta$ -decay spectra are due to a similar case of quantum mechanical interference. According to Fermi's golden rule, the  $\beta$ -emission intensity is essentially proportional to the probability of finding the  $\beta$ -particle at the nucleus. From the laws of quantum mechanics, that probability is the square of the  $\beta$ -particle wave-function, which in a solid consists of two interfering amplitudes: an outgoing wave from the  $\beta$ -emitter; and a wave scattered back to the nucleus from nearby atoms. It is the constructive (or destructive) interference of these components that leads to increased (or decreased) emission. The oscillations arise because the phase shift between the outgoing and backscattered amplitudes is energy dependent: the phase shift is approximately given by the geometrical path length divided by the de Broglie wavelength  $h/p$ , where  $h$  is Planck's constant and  $p$  the particle momentum. Indeed, the oscillations in BEFS and EXAFS are identical except for a small



TILTING a thin wafer of lanthanum aluminate at  $45^\circ$  is the secret behind this picture, which has won the grand prize in Polaroid's instant photomicrography competition. Michael Davidson found that shooting the superconductor substrate at an angle revealed a "stairstep twinning" in its structure that had been previously invisible. **C.A.**



## RÉSUMÉ

phase shift and a 180° phase reversal, attributable to selection rules.

Thus Koonin's idea may have a practical importance which transcends the massive-neutrino controversy. That is, BEFS can also be used as a probe of local atomic structure, yielding both interatomic distances and coordination numbers. The great advantage of EXAFS-type probes, compared with techniques like X-ray diffraction, is that crystalline samples are not needed. Moreover BEFS does not require a synchrotron X-ray source, although long count times may be needed to obtain an adequate signal-to-noise ratio. Koonin gives several potential applications, together with approximate calculations of the size of the effect. For example, with tritium as a  $\beta$ -emitter, one could map the locations of hydrogen in solids, a feat which is difficult with other techniques, because hydrogen is a weak scatterer. Interestingly, the calculations of BEFS were all made using scattering amplitudes and phase shifts from Koonin

and Meredith's computational recipes<sup>4</sup>.

As for the heavy neutrino, the earliest evidence of this came from the  $\beta$ -decay of tritium implanted in germanium<sup>5</sup> which gave a spectrum with small departures from that predicted at the high-energy tail. Koonin also cites evidence from frozen molecular tritium experiments. In each case, the BEFS effect would have a magnitude comparable to the observed effect, although Koonin does not believe it gives the whole explanation. And experiments with sulphur and germanium isotopes, in which the  $\beta$ -decay energy is much greater, would not be affected by the BEFS effect, according to Koonin. □

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## MOLECULAR BIOLOGY

## Signal recognition revisited

Sean Munro

DISCONCERTING but true — deletion of the budding-yeast homologue of a gene encoding a protein involved in some seemingly crucial cellular process does not necessarily result in the death of the yeast cell. A further surprising example of this phenomenon is described by Hann and Walter in *Cell*<sup>1</sup>. They find that budding yeast, *Saccharomyces cerevisiae*, contains a 'signal recognition particle' but that the particle is not essential for the organism's viability.

The signal recognition particle (SRP) helps proteins destined for export from the cell, or incorporation into membranous compartments, either to cross or to become incorporated into a lipid bilayer. In eukaryotic cells this involves insertion of the protein into the membrane of the endoplasmic reticulum<sup>2,3</sup>. The proteins are made with a hydrophobic signal sequence which can be removed once translocation has begun. The signal sequence is recognized by SRP, the binding of which inhibits further translation by the ribosome until the resulting complex docks onto a protein (the SRP receptor) in the membrane of the endoplasmic reticulum. At this stage, SRP is released and the nascent chain is passed to a translocation complex, or 'translocon', and translation resumes with the polypeptide cotranslationally passing through the membrane of the endoplasmic reticulum.

Thus SRP provides an elegant way of ensuring that proteins destined for the

secretory pathway are not translated prematurely in the cytoplasm. It is assumed that this is desirable because such proteins might either fold too tightly to be subsequently threaded through the translocon or would fold abnormally and so aggregate.

The SRP is a complex of a short RNA molecule (7SL) and six proteins, the best characterized of these being SRP54 which binds to the signal sequence as well as to 7SL RNA<sup>2,3</sup>. Well-conserved homologues of the components of SRP have been found in a range of eukaryotic organisms<sup>3</sup>, and two groups independently isolated a gene encoding an SRP54 homologue from *S. cerevisiae* and concluded that the gene was essential for viability<sup>4,5</sup>. All of this seemed consistent with SRP's central part in the secretory pathway.

Other reports were, however, at odds with this simple story. Studies *in vitro* showed that some small yeast proteins, notably prepro- $\alpha$ -factor, could cross the membrane of the endoplasmic reticulum post-translationally<sup>6,7</sup>. And biochemical and genetic studies in yeast demonstrated a requirement for members of the 70K heat-shock protein (hsp70) family for efficient translocation of particular proteins, including prepro- $\alpha$ -factor, into the endoplasmic reticulum<sup>8,9</sup>. These results could be explained by arguing that some small fraction of proteins are able to use hsp70 and other factors to get into the endo-

### Off the shelf

MAKING monoclonal antibodies without the use of animals is a practical reality, report J. D. Marks *et al.* (*J. molec. Biol.* **222**, 581–597; 1991). They show how antigen-specific antibody fragments can be selected from a library of recombinant immunoglobulin genes, which were cloned from an unimmunized human donor and expressed on the surface of bacteriophage. The library was the key to success — with some  $2 \times 10^7$  members it is similar in size to the B-cell repertoire of the mouse. Avoiding immunization *in vivo* obviates the problems associated with immunizing humans to order, and allows antibodies for many different antigens to be isolated from a single library. Immunizing animals does however provoke the selection of high-affinity antibodies that have undergone somatic mutation, and mimicking that process is the next challenge.

### G whizz

RESULTS from the deep northeast Pacific pour cold water on the notion that there is a mysterious 'fifth force' (M. A. Zumberge *et al.* *Phys. Rev. Lett.* **67**, 3051–3054; 1991). Discrepancies in gravity measurements from a mineshaft, and others revealed in Eötvös's legendary laboratory determinations of big G, Newton's gravitational constant, hinted that all was not well with Newton's inverse-square law of gravity. The fifth force was intended to resolve the differences. In measuring the gravitational acceleration down to depths of 5 km under water, the new tests resemble the mineshaft experiments (conducted to 1-km depth) but avoid the dangers from unseen variations in density in the surrounding material. The measured value of big G, with a resolution of 2%, agrees to within 1% with laboratory measurements, so removing any requirement for a fifth-force correction.

### Injury and Alzheimer's

THE report of a new mouse model for Alzheimer's disease (see pages 432 and 476 of this issue) follows hard upon a paper in last week's *Lancet* in which G. W. Roberts *et al.* describe an association between severe head injury and the deposition of the  $\beta$ A4 amyloid plaques characteristic of the disease. Following up their own hypothesis, Roberts and colleagues looked at the brains of 16 people who had died after suffering trauma to the head. They found abnormally large deposits of plaque had developed in the brief (6–18-day) interval between injury and death in six of the 16, and say that the study lends support to the view that head injury can result in the pathological  $\beta$ A4 response also deeply implicated in Alzheimer's disease.

plasmic reticulum post-translationally and so would not require SRP to ensure cotranslational translocation. However a startling discovery was made following the cloning of a candidate for the *S. cerevisiae* homologue of 7SL<sup>10</sup>. This RNA molecule, scR1, does not have a size and sequence typical of a 7SL but its abundance and intracellular localization are consistent with such a role; yet scR1 is not an essential gene. So either it is not part of the SRP or the SRP is not actually essential in *S. cerevisiae*.

This issue has now been resolved by Hann and Walter<sup>1</sup>. They initially used antibodies against *S. cerevisiae* SRP54 to demonstrate that it was in a complex with scR1 and showed that deleting the scR1 gene prevented SRP54 assembling into a large particle, confirming that scR1 is the *S. cerevisiae* 7SL homologue. They then went back to their yeast without the SRP54 gene and discovered that they were not in fact dead, but rather were growing at a considerably reduced rate. Furthermore, deleting the genes for both scR1 and SRP54 resulted in the same slow growth rate as either deletion alone.

From this and the failure of low-stringency probing to reveal any genes related to SRP54 and scR1, Hann and Walter conclude that there are no structural homologues which can substitute in the deletion strains. This is a reasonable assumption and leads to the remarkable conclusion that in *S. cerevisiae* every protein that needs to get into the endoplasmic reticulum can do so without the help of SRP. Analysis of five such proteins in the SRP54-deficient strain revealed that all do indeed get into the endoplasmic reticulum, but with very different efficiencies. For some, more than half the protein made remained in the cytoplasm, whereas others were translocated as efficiently as in wild-type cells. Interestingly, when SRP54 was removed from cells by placing the gene under the control of a regulated promoter and then switching it off, translocation into the endoplasmic reticulum was initially more severely impaired than in the strains growing without the gene at all. This suggests the induction of other proteins to facilitate long-term growth without SRP.

The survival of yeast without SRP does not mean that it is an unimportant part of the secretory pathway; the yeast without it grow extremely slowly and in the fission yeast, *Schizosaccharomyces pombe*, 7SL does seem to be truly essential. What it does mean is that there must be a way for proteins to translocate into the endoplasmic reticulum without SRP to ensure that it is cotranslational. One possibility is that signal peptides on nascent chains can dock directly into the translocon so long as translation has not

proceeded to a point where folding obscures the signal or prevents translocation. Alternatively the hsp70-dependent post-translational mechanisms may come into play, with hsp70 or other chaperones not only keeping the proteins in a soluble state but also stabilizing partially folded intermediates which would have the ability to unfold for translocation. Indeed, the level of heat-shock proteins is elevated in the SRP54-deficient yeast, although this may just be in response to the presence of abnormal proteins in the cytoplasm.

This now raises the question of how much these post-translational mechanisms using general chaperones contribute to protein translocation in normal cells. In the prokaryote *Escherichia coli*, many proteins are exported across the inner membrane post-translationally with the aid of the Sec B chaperone protein<sup>11</sup>. Although Sec B is not essential in this process, viability without it requires activation of the heat-shock response<sup>12</sup>. *Escherichia coli* also has an SRP-like particle, but given the considerable amount of post-translational translocation it may have only a minor involvement in protein export<sup>13</sup>.

It may be that all organisms have several systems for cotranslational or post-translational translocation of proteins, all of which converge on a common translocon in the relevant membrane. During the course of evolution different proteins may have become more or less optimized to use of a particular system, with the result that the relative ratio by which these systems are used varies between different proteins in the same organisms and at a bulk level between organisms. However that may be, the study of protein translocation is likely to have much scope for debate as well as much to tell us about general mechanisms for protein folding and assembly. □

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## Original gravity

MOTION sickness makes no biological sense. What possible evolutionary advantage can primitive man have gained from the disabling nausea and vomiting brought on by violent movement?

Daedalus comments that motion sickness is a product of sensory conflict. It arises when our eyes and limbs, judging our position and acceleration, find themselves contradicted by the balancing organs of the inner ear. In this connection he recalls that both strong alcohol and heavy water can bring on dizziness and vomiting when swallowed. They enter the bloodstream, diffuse into the canals of the inner ear, and — because they differ in density from the fluids inside — set up strange and dizzying convection currents.

Primitive man is unlikely to have encountered heavy water. But he may have come across strong alcohol. Most modern brewery yeasts produce a weak solution containing only 6–7 per cent alcohol. But suppose some enterprising yeast of the distant past, flourishing on honey, fruit and other sugar-rich sources, produced a juice containing (say) 50 per cent alcohol? Our ancestors might well have rushed to consume the product, to their ultimate regret and downfall. Helplessly drunk, the old man of the tribe could easily have been deposed by his younger rivals; nursing a throbbing hangover the morning after, he would have been easy prey for sabre-toothed tigers and similar predators. Female palaeo-alcoholics would have had fewer and less successful children. Hence, says Daedalus, the strong evolutionary advantage of the vomiting reflex. By ejecting the stomach contents whenever the inner ear gave strange, discordant signals, it saved our ancestors from the Demon Drink.

This elegant theory also explains the evolution of our other defence against alcohol — a liver which can destroy it at a staggering 10 grams an hour, far in excess of any known natural requirement. Our love-hate relationship with alcohol must go back a long, long way.

So Daedalus proposes an expedition to the African jungles in which our kind evolved, to search for that wild primeval yeast that gave us alcoholism and motion sickness. Once identified and cultivated, it could generate a range of new and ferocious fermented drinks, as potent as distillates like vodka. Brewers will rejoice — and so will astronauts. In micro-gravity their inner ears are, of course, free of all convection currents. Once acclimatized to this unique state, they should, according to Daedalus's theory, be able to enjoy the delights of the strongest alcohol, or even heavy water, with no queasiness at all. David Jones



# Galactic halo gamma rays

SIR — Recent reports<sup>1,2</sup> on the isotropy of the 117 observed and located  $\gamma$ -ray bursts (GRBs) and the  $V/V_{\max} = 0.34 \pm 0.03$  value for them has far-reaching consequences.  $V/V_{\max}$  is the average over the bursts of  $(C_p/C_{\lim})^{-3/2}$ , where  $C_p$  is the peak count of a burst and  $C_{\lim}$  is the limiting count that triggers detection<sup>3</sup>. For a spherical distribution of sources with a luminosity function that is spatially invariant,  $V/V_{\max} < 0.5$  when their density drops outwards. Thus, the observed value suggests that the distribution of sources is either local around the Solar System, or local around the Milky Way, or of cosmological dimensions.

I would like to consider the possibility of a distribution of about 50 kpc around our Galaxy which, for this number of bursts, is not excluded by the reported lack of clustering in the direction of the Large Magellanic Cloud, in view of its relatively small mass, or by the Sun's off-centre position. Because galactic escape speeds are of the order of 300 km s<sup>-1</sup>, any objects coming from the Galaxy would occupy a far larger volume, unless their velocity is finely tuned or unless they are able to produce only GRBs during a limited, well-selected epoch of their lifetime. Otherwise, we must assume that the distribution is pregalactic. Unless very different from galactic ones in origin or in ability to produce GRBs, pregalactic neutron stars would be associated with too much supernova debris to be compatible with the chemical composition of the Galaxy, if their number greatly exceeds that of the former; if they are less numerous, it is not clear why no trace of the galactic distribution was observed.

With the reported detection threshold, maximum intrinsic luminosities in the case of a  $\sim 50$  kpc distribution are several  $10^{39}$  erg s<sup>-1</sup>. We have suggested that accreting, rapidly rotating black holes are the sources for at least some of the GRBs<sup>4-6</sup>. An attractive feature of this model was that its Compton-Penrose spectra, characterized by the electron rest-energy, were similar to those observed. These spectra are generated by Compton scattering in the inner ergosphere of photons that are emitted by the infalling matter and are travelling inwards. On arrival at the inner ergosphere they become extremely blueshifted in the local inertial frame and come out of the collisions with the locally orbiting electrons having energies around the electron rest energy. The very fast black-hole rotation then counteracts gravity on these photons and, if the optical depth is right, they emerge to infinity with negligible redshift.

I now wish to point out that if the 'dark matter' halo of our Galaxy consists of  $\sim 1\%$  (by total mass)  $\sim 10^{-13} M_{\odot}$  asteroids and if the bulk of the mass is in  $\sim 10 - 30 M_{\odot}$  black holes rotating very close to the extreme Kerr limit, then an asteroid will come to within a Roche lobe distance of a black hole about once a day, the observed GRB rate. In this encounter the asteroid would become bound, would break up and would accrete onto the black hole to produce the GRB when matter hits the inner ergosphere of the black hole. Asteroid-asteroid collisions would not significantly deplete that reservoir.

This particular composition of the halo could obtain if the fragmentation process in the pregalactic halo terminates with  $\leq 30 M_{\odot}$  collapsing clouds, of which the central part eventually becomes a black

hole but the exterior, which acquires metallicity from the nucleosynthesis, forms a disk to take up the excess angular momentum. The disk then fragments into the asteroids, some of which subsequently become unbound.

With the increased sensitivity of future X-ray detectors, such black holes, if they exist, could be seen as they pass through the galactic disk with galactic escape speeds and accrete interstellar clouds.

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## AIDS response

SIR — Maddox<sup>1</sup> and Anderson<sup>2</sup>, discussing the results of Stott *et al.*<sup>3,4</sup> appear to differ on the key issue of whether or not the xenoreactive monkey sera bound SIV-encoded proteins (although Stott *et al.*<sup>4</sup> reported no such reactivity was detected with purified envelope proteins). But both authors<sup>1,2</sup> implicate some protective cross-reactive immunity between SIV-encoded proteins and cellular (presumably histocompatibility) antigens.

This interpretation overlooks at least two readily testable alternative explanations of the phenomena described. First, HIV budding from a host cell might incorporate cell-surface molecules, providing both the initial xenoimmunizing and subsequent neutralizing epitopes. Second, immunization with cellular components from a CD4<sup>+</sup> line might induce anti-CD4 antibodies which could block subsequent viral access to CD4<sup>+</sup> cells. Watanabe *et al.*<sup>5</sup> have demonstrated just such an anti-self CD4 mechanism in rhesus monkeys protected from challenge with SIV following soluble CD4 treatment.

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SIR — Stott *et al.*<sup>4</sup> present evidence that antibodies against proteins of human lymphoblastoid cells in sera from macaques immunized with fixed, uninfected cells (or partially purified, inactivated whole SIV) might correlate with protection from infection after challenge with live cell-free SIV grown in the same human cells. This raises the possibility that vaccine protection in macaques immunized with SIV vaccines might be

influenced by immune responses to cellular proteins, as the vaccine and challenge viruses used in previous studies were grown in human cells<sup>4</sup>. In this regard, reports describing vaccine protection against SIV infection in macaques have not included an analysis of antibodies against cellular proteins.

We have been evaluating antibody responses in rhesus macaques immunized with whole, inactivated SIV, and subsequently challenged with live cell-free SIV. The vaccine and challenge stocks of SIV were grown in the human lymphoblastoid cell line CEM $\times$ 174. Virions used for vaccination were purified by centrifugation through a glycerol bed and inactivated with psoralen and ultraviolet light. After intravenous challenge with live cell-free SIV (50 MID<sub>50</sub>), all 10 animals that received the SIV vaccine were protected from infection, whereas all 6 animals immunized with HBsAg (as a control vaccine) became infected<sup>6</sup>. While performing assays for neutralizing antibodies on plasmas from these animals, we noticed abnormal cell clumping and growth inhibition of CEM $\times$ 174 cells at dilutions approaching the neutralizing titre. These effects were observed in plasmas from SIV-vaccinated animals but not in plasmas from HBsAg-vaccinated animals, prompting us to suspect that anti-cell antibodies were present in the SIV vaccinees.

To characterize further the anti-cell antibodies, we performed the following experiments. First, we showed that anti-cell antibodies were present in plasma from the SIV vaccinees by using a fixed-cell (H9 and CEM) immunofluorescence assay. In contrast, no anti-cell antibodies were found in plasmas from HBsAg vaccinees or, importantly, macaques that had been experimentally infected with

SIV grown in human cell lines. Similar results were reported by D. P. Bolognesi *et al.* (IV Int. Conf. Adv. AIDS Vaccine Dev., Marco Island, 1991) who, in addition, found no anti-cell antibodies in monkeys vaccinated with recombinant vectors or subunit vaccines. Second, we absorbed out a significant portion of the anti-cell antibodies by incubating plasmas with uninfected CEM $\times$ 174 cells. Removal of the anti-cell antibodies reduced the cell clumping and growth inhibition, but did not affect the *in vitro* SIV neutralizing titre. Third, we examined plasmas from the SIV vaccinees for the ability to block binding of monoclonal antibodies (OKT4a and OKB7) to the surface of CEM $\times$ 174 cells, as measured by flow cytometry after live-cell fluorescence staining. Our findings indicated that the anti-cell antibodies present in the plasmas did not interfere with the ability of OKT4a to bind to CD4, or of OKB7 to bind to CR2 (complement receptor type 2). Finally, we tested the ability of the anti-cell antibodies to block live SIV from binding to CD4-bearing human T cells and found that SIV binding was not inhibited. Thus, these data suggest that the anti-cell antibodies elicited in response to our whole SIV vaccine preparation do not appear to play a major role in the *in vitro* neutralization of SIV. Furthermore, it appears that the anti-cell antibodies do not block access of SIV to the surface of target cells.

We should like to mention some other points. Exposure of macaques to the human CD4 protein elicits an antibody response to the human molecule that is capable of blocking SIV infection<sup>5</sup>. Thus, in macaques immunized with uninfected human CD4<sup>+</sup> cells, a similar antibody response and effect might explain the observations of Stott *et al.* In terms of protection conferred by immunization with infected cells, we should note that CD4 expression in infected cells is markedly reduced when compared to uninfected cells. Therefore, it seems likely that specific immune responses to SIV antigens are important in the protection against challenge infection. Of course, we cannot exclude a combination of both mechanisms. Additional evidence for the role of an SIV-specific immune response in protection is found in the work of Putkonen *et al.*<sup>7</sup>, who showed that passive transfer of pooled serum from experimentally infected macaques to naive macaques provided protection from challenge infection. Because we did not find anti-cell antibodies in plasmas from our experimentally infected macaques, we assume that none were present in the macaques of Putkonen *et al.*<sup>7</sup>. Taken together, these data suggest that SIV-specific immune responses do provide protection, but that

additional means (for example, CD4 immunization) of inducing protection should be considered in an overall vaccine strategy for AIDS.

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SIR — Maddox's otherwise helpful article<sup>1</sup> began and ended unfortunately. I fear that the structure of its assessment of Hoffmann's and Stott's work is going to yield all sorts of bizarre press reports, leading to confusion among the general public.

As I understand it, Duesberg essentially finds the lack of fit of HIV into Koch's and Rivers' postulates as grounds to dismiss HIV as significant in the aetiology of AIDS. Indeed, HIV doesn't fit these guidelines, and the more that is learned about it the more evident it becomes that just as Koch's postulates had to be modified by Rivers to account for the differences between bacterial and viral pathogenesis, so it may be necessary to devise new postulates to describe the relationship between lentiviruses, even all retroviruses, and their hosts.

Basically, Duesberg's objection is that the pattern of HIV-host interaction is

not yet clearly delineated, and that no obvious, standard, known pathobiological mechanism easily succeeds in establishing HIV as the aetiological agent of AIDS. Just as autoimmunity figures in many human diseases, such as the production of cross-reactive antibodies in response to  $\beta$ -haemolytic streptococci which subsequently mediate rheumatic carditis, so may the reports by Kion and Hoffmann<sup>8</sup>, and by Stott *et al.*<sup>3</sup>, lead to a picture of AIDS pathology involving an autoimmune process triggered by HIV. Novel aspects of immune function may well be part of HIV-mediated pathology<sup>9</sup>, and viral latency further complicates fitting HIV into standard pathobiological models<sup>10</sup>.

Maddox wrote as much in his article, yet the opening and closing sentences are what the popular press will notice. This causes confusion for all concerned: false and misleading hopes for those directly engaged with the disease, and weeds to be uprooted by those of us involved in education.

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## Efficiency in a liquid solar cell

SIR — We have demonstrated<sup>1</sup> the effect of cyanide on CdSe ferrocyanide photoelectrochemical cells (PECs), resulting in the highest solar-to-electrical conversion efficiency for a PEC. It has been suggested that 26% of the observed photocurrent was due to a "virtual battery" resulting from electrochemical discharge of cyanide to cyanate at the photoelectrode<sup>2</sup>. We do not observe this battery effect, and our investigations indicate that results in ref. 2 supporting the effects are artefacts of the experimental configuration in that study.

Specifically, in a conventional PEC, without storage<sup>3</sup>, the electrochemical reactions occur within a single cell. However, the "battery effect" study used a separate anode/cathode compartment cell<sup>2</sup> which artificially concentrates ferricyanide in the cell. This exaggerated ferricyanide chemically reacts with cyanide creating the reported cyanate buildup. Our further experiments indicate that no cyanide is electrochemically oxidized at either CdSe or at Pt electrodes. A simple non-electrolysis dark

cell shows that all cyanate is due to chemical and not electrochemical origin and hence is not related to photocurrents. The rate at which cyanide and ferricyanide react is controlled and minimized by solution modification techniques<sup>4</sup>, which optimize speciation in solution. Finally, our repeated studies show identical photocurrents for a properly prepared CdSe ferro/ferricyanide cell both with and without added cyanide. The contention of different quantum efficiencies without cyanide<sup>2</sup> is not reproduced. Interested readers can find expanded details of this correspondence in ref. 5.

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# Adopting a new culture

R. G. W. Anderson

**The Study of Change: Chemistry in China 1840–1949.** By James Reardon-Anderson. Cambridge University Press: 1991. Pp. 444. £45, \$59.95.

THE chemistry of the Western world reached China only in the middle of the last century. Before that, the Chinese had different and individualistic systems of chemistry, the development of which has been assiduously traced by Joseph Needham and his team in the fifth volume of *Science and Civilisation in China* (Cambridge University Press; more than 4,000 pages so far). James Reardon-Anderson, of Georgetown University, has gathered a great deal of information on the period when Westerners brought their chemistry to China, when Chinese scholars started to study in the West and returned home with new knowledge, and when institutions of higher education developed in China for educating and training on home territory.

The period under review, 1840 to 1949, encompassed important upheavals. From 1644 until 1911, China was ruled by emperors of the Ch'ing Dynasty. The Sino-Japanese War of 1894–95 weakened traditional structures and this led to the revolution of 1911. There followed an unstable interregnum until 1927 when the nationalist government under the Kuomintang Party took charge. The subsequent period, the Nanking decade (so called because the party made Nanking its capital), was characterized by strong central authority. In 1937 the Japanese invaded and the nationalists, who retreated inland, gradually lost power to the communists. By 1949, after four years of civil war, the Chinese Communist Party was left firmly in control. Reardon-Anderson successfully relates the development of chemistry in China to these different political climates. He stimulates thoughts about how our own scientific development may have been influenced by changing political forces, and about how even the most academic pursuits cannot be charted accurately by applying purely internalist historiography.

The Chinese did not take to Western chemistry naturally. The classically trained scholar was characterized by "bookishness, the pre-occupation with philosophical and literary subjects, denigration of manual and technical skills, and undue respect for established authority". Libraries were preferred to workshops. There were, however, changing currents being felt in the middle of the nineteenth century, at the very time when contacts with the West were increased. Hsü Shou, who failed his Civil

Service examination, met John Fryer, an unconvinced English missionary, in 1867 at the Kiangnan Arsenal. Here they established a translation bureau and, by 1908, 139 science books had been published, the first chemistry tract being David Ames Wells's *Principles and Applications of Chemistry* (which, when translated back from Chinese, becomes the more enticing *Authentic Mirror of Chemical Science*).

An immediate problem was nomenclature. Although the Chinese had names for some of the metals, new scientific terms were burgeoning and the Chinese language did not lend itself to an easy solution. All agreed that only Chinese characters should be used — there would be no borrowing from the Roman script. Translators also decided that Western sounds should not be transliterated. Ultimately, the main figures involved in this labour agreed that a single character should be assigned to each chemical element. After much consideration, Fryer and Hsü published their *Chinese–Western Glossary of Chemical Substances* in 1885. It was a necessary step for any future progress, although a system for organic chemistry did not come until later.

Institutionalization of science started early in the twentieth century with the establishment of mission colleges and other bodies for teaching at an undergraduate level. The Rockefeller Foundation played a large part, with surveys of medical-education provision in 1914 and 1915, followed quickly by generous funding of the Peking Union Medical College. This and other colleges were staffed by US teachers of high calibre, which led to the better Chinese students going to the United States to obtain postgraduate qualifications. Chinese schools did not prepare students well for higher education in science. Instruction was almost entirely by lecturing and little practical work was attempted. Teachers sometimes taught under misconceptions caused by problems with translation: a botany teacher of the 1920s

once misread the characters for 'natural conditions' as 'heavenly dragon conditions' and the lesson went off at a tangent, the teacher lecturing the class on the difference between the flying dragons of the heavens and the manifest dragons of the fields.

In spite of the cultural difficulties, serious and scholarly chemists did emerge from the system. The most prominent of the first generation was Wu Hsein, who graduated from Harvard Medical School and the Massachusetts Institute of Technology. As a biochemist, Wu's early work was to devise techniques for measurement of constituents in the blood (the Folin–Wu method). Later he developed a theory to explain denaturation of proteins. Because of the sympathetic environment of the Peking Union Medical College, his reputation grew and he came to be regarded as a member of the international scientific community. But the dependence of Chinese graduates on the West, in that they had to travel to the United States and Europe for their postgraduate education, did have a deleterious effect. The best graduates lost touch with students who were immediately junior to them, and whom they would have been helping to teach had they stayed in China. This task was assigned to less able laboratory instructors, who had no prospect of promotion.

One of the most contentious areas of



Oil-laden desert in Kuwait. Reproduced from *Tides of War: Eco-disaster in the Gulf* by Michael McKinnon and Peter Vine, a graphic and dramatic account of the ecological disruption ensuing from the Gulf conflict. Published by Boxtree, price is £16.99.

debate in the development of a Western-style chemistry community was how research topics were chosen. By inclination, the Chinese preferred pure rather than applicable research. Inevitably there were tensions between the needs of the nation and the predilections of the chemists. Until the end of the Nanking decade, chemists were not especially pressured to develop practical solutions to problems of Chinese industry. But when the communists gained greater influence, life became more difficult for those who wanted to conduct only pure research. A chapter dealing with the almost overwhelming difficulties of running a chemical industry in isolated areas of China during the Japanese invasion makes clear the urgent national need that was felt for chemists to descend from their ivory towers. Later on, life was made very hard for pure scientists for ideological reasons. The point is made that, in the West, scientists were quite prepared to turn their hands to practical problems in time of war. In China, this was not the case, and feelings ran exceedingly high. There were extreme responses. Chemists were later to be tortured and killed by the Red Guards for adopting unacceptable attitudes, or simply for being regarded as an élite class.

Reardon-Anderson provides a wealth of information about a fascinating process. Not infrequently, the detail becomes too dense, and it is difficult to follow the thread of his argument. Attention is constantly arrested by contrasts with the West: before 1920, fewer than 30 Chinese in the world possessed doctorates; before 1928, no Chinese university had a medical school; although agricultural improvements were a clear priority, only one paper of the 442 research articles published in China in chemistry between 1929 and 1949 dealt with agricultural chemistry; only 411 metric tons of sulphuric acid (used traditionally as an indicator of industrial development) were produced in nationalist China in 1941.

The book is not a straightforward history of science: it is as much economic and social history. Reardon-Anderson pleads that nonscientific sinologists should study the volume, claiming that "Science is not that hard!" (although he makes some slips — antiseptic agents not antibiotics, were first used by Lister in 1865, and what is "ammonium gas"?). The book is a difficult but worthwhile read for those who are interested in the transfer and absorption of a new scientific culture, and particularly in the relationship between science and the state. □

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## Pornophilosophy

Richard Dawkins

**Mystery Dance: On the Evolution of Human Sexuality.** By Lynn Margulis and Dorion Sagan. *Summit Books*: 1991. Pp. 224. \$19.95.

LYNN Margulis is a distinguished biologist: a courageous outsider whose eventual hard-won triumph constitutes one of the important scientific revolutions of our age, the startling realization that our cells are nothing less than symbiotic colonies of bacteria. It would have been good to have heard her views on "the evolution of human sexuality", the subtitle of this book.

As for what we actually get, I imagine that she is less to blame than her coauthor (and son) Dorion Sagan. Indeed, other evidence implicates him as a sucker for those trendy French 'philosophers' whose influence liberally, irrelevantly and distractingly pervades — indeed, ruins — the book. Their 'philosophy' avowedly consists in playing games, which might seem harmless enough. But these charlatans want it both ways — to enjoy a self-indulgent frivolity while simultaneously being thought profound.

Had you noticed that the French word *lit* means both 'read' and 'bed', *hein*? *Tiens, quel joli* joke. Even better, "*semantics* and *semiotics* bear an evocative resemblance to the sexual word *semen*". Oh, such formidable deconstruction, is it not? And "the English verb *mean* shares roots with *moan*" (nudge nudge). But attend, I can cap (wink wink) that trope there. At my school the slang word for erection was — but no I cannot bare it, it has such a droll signification — 'root'.

What on earth have these puerile self-confessed games to do with anything? There are several thousand languages in the world, a rich statistical sample. Has the French 'philosopher' done a systematic survey of them to test his hypothesis that words about sex and about meaning are connected? Margulis the tough-minded scientist would demand no less when refereeing a scientific paper. But *chic savants* can apparently indulge in idle tossing off and get away with it. I mustn't be too high and mighty, though: I recently heard a lecture by an Oxford disciple of this school of modish French dippiness, and it took me a full five minutes to see through him (it was when he made his big point about Jesus's name and Jacques Derrida's both beginning with J).

The philosophical priorities in this book can be gauged from the authors' bizarre estimation of Heidegger as

"perhaps the most influential philosopher of the twentieth century". He was the old Nazi, you remember, who single-handedly wrestled with the problem of 'being'. Without Heidegger it would have escaped us that "The nothing annihilates itself". Even Lacan (a favourite among Margulis and Sagan's French mentors) refers to Heidegger's "dustbin style in which currently, by use of his ready-made mental jetsam, one excuses oneself from any real thought".

The book is built around a recurring theme of striptease. The androgynous stripper peels off garment after garment, metaphorically revealing our evolutionary past. S/he is forever twiddling round and changing sex — sorry, "gender" — on successive gyrations of the mystery dance. At times the language suggests a cack-handed attempt at eroticism. Or it may seek to embarrass, in which case it succeeds:

As she climaxes a man briefly appears beneath her, existing only long enough to ejaculate before disappearing again into her shuddering loins [apologies to W.B. Yeats not offered]. In retrospect the audience realized that they saw her give ejaculatory virtual birth to a full-grown male. The body turned and below the rippling abdomen of the seven-veiled striptease artist was an obscure pubis, dark and hairy. The erect penis, the unmistakable signal of his sex, shrunk on the next rotation. It became the clitoris [pp. 59–60].

Yuck! But there's more:

She lustily stands spreadeagle. Slowly she turns and bends to expose her dark buttocks and the wet genitals below, bidding him welcome [p.60].

These passages are chosen more or less at random. The pages drip with similar 'pornophilosophy', a genre which, I understand, originated with Sartre. Personally, if I must have porn, I prefer it shorn of pretension. One cannot help feeling that if Desmond Morris had written that paragraph (he wouldn't) he'd be noisily accused of flagrant, pot-boiling sensationalism. Should this book get away with it just because of who Lynn Margulis is?

Actually, although it has a joyless earnestness where Morris has a teasing sense of fun, *Mystery Dance* in one way approaches *The Naked Ape* — the same charmingly cavalier lack of evidence for the same daringly speculative functional explanations. In some cases literally the same explanations, albeit unacknowledged. Margulis and Sagan attribute one of their theories of the female orgasm to an unpublished correspondent, a businessman inspired by a conversation with a sexually boastful US airman. In fact, as I told the same businessman when he wrote to me some years ago, an identical theory was clearly



set out in the *The Naked Ape* (distributed around the world in a mere 12 million copies).

Meanwhile, back at the dance, more and more veils are lasciviously removed until we expose the ancestral bacteria. Ah, you think, now Margulis must have something worthwhile to say. But no. With a deft gyration of the intellect, our scientific authoress rotates herself into her literary *alter ego* and, by the time he has given vent to another stupefying salvo of continental obscurantism, the moment has passed:

But there is perhaps a deeper phase, the metaphysical plane of pure phenomena, continuous appearances. The evolutionary stripper is a curious creature: the G-string is not a thin cloth decorated with tassels but rather a word, a letter, a musical symbol for ultimate nakedness [honestly, I'm not making this up]. Paradoxically, when the G-string is removed — to the accompaniment of strange vibratory music consisting in part of a silent triangle and a gentle crash of cymbals — the nakedness is gone. S(he) stands before us as fully dressed as ever before [p.27].

What on earth, you may say, is all this about? Well, wait:

We encounter our sexual ancestors along the slippery slope of signs and signifiers, via the medium of language. The use of signs of any kind necessarily obscures; words represent or replace signified things in absentia; they are little black masks. We postpone reality to discuss it; without this postponement, this instantaneous replacement of our sexual ancestors, or things in general, by their signs there could be no possibility of language, of signification at all [p.28].

And then where would we be?

How a scientist of Margulis's calibre and rigour can be gulled by this pretentious drivel is as far beyond comprehension as the prose itself. Let us be charitable and hope that she had an argument with her coauthor and lost. But if, rightly, you value Lynn Margulis and her reputation, do her a favour and ignore this book. □

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## Paths of least resistance

Andrew Holmes-Siedle

**Semiconductor Devices: Pioneering Papers.** Edited by S. M. Sze. *World Scientific*: 1991. Pp. 1028. \$112, £65 (hbk); \$68, £39 (pbk).

SEMICONDUCTORS have been single-handedly responsible for the miniaturization of electronic circuits, which has reduced the cost and increased the reliability of electronic components, and, more importantly, reduced signal-propagation times. With integrated circuits, the entire circuit, or group of circuits, is etched on one chip of semiconductor material. The immense computing power that can arise from the logic circuits on a silicon chip has been the basis of the computer revolution that has so drastically changed our lives.

From the beginning, devices that depend on semiconductors have been developed with both military and non-military applications in mind. First came radio and radars. Later came calculators and missile-guidance computers, then medical instruments and 'smart' weapons. Today semiconductor devices are familiar to almost everyone as essential components of solar-power equipment and personal computers.

A semiconductor is a material, such as silicon or germanium, having a resistivity between that of conductors and insulators and whose resistance decreases with rising temperature and doping — the inclusion of impurities. Phosphorus as an impurity in silicon,

increases the supply of electrons that participate in conduction, giving an n-type semiconductor (conduction predominantly by negative charge carriers). Boron as the impurity increases the conductivity by the introduction of 'holes' — vacant sites into which electrons can move. This gives a p-type semiconductor, where conduction is predominantly by holes that are equivalent to positive

charge carriers. Junctions between semiconductors of different types form a fundamental part of modern electronic components and circuits: p- and n-type semiconductors in contact give a rectifier, where the resistance in one direction is much greater than in the other. Two such junctions placed back to back produce a bipolar transistor, which can amplify currents and be used in switches.

Sze has now compiled 141 of the most important research papers on semiconductor devices dating back to the early years of this century. He divides the book into four sections, each covering a different category of device — bipolar, unipolar, microwave and photonic. In bipolar devices, both electrons and holes play an essential part, whereas in unipolar devices, such as the field-effect transistor, current flow is due to the movement of one of the types of charge carriers. Modern transistors are mainly bipolar devices. They do most of the jobs that electron tubes ('radio valves') did and are cheaper, smaller, lighter, easier to manufacture, more widely applicable and more efficient.

To make a good semiconductor device, it is essential that much of the underlying physics is understood. For bipolar devices, most of the early work was centred on the action of the p-n junction, the region at which two dissimilar types of semiconductor meet. This structure was a latecomer in the development of electronics, and its inception is recorded in this volume by a paper first published in 1949. Other rectification methods, such as 'point contacts', were used up to this time. The earliest paper in the book is on unipolar devices and dates back to 1874.

We also learn about the early days of radio in the 1920s, when a simple detector, called the crystal, was used. In this radio receiver, a 'cat's whisker' wire was used to make contact with the crystal semiconductor. Certain materials in contact with one another were known to possess a rectifying action, and copper oxide rectifiers (copper in contact with cuprous oxide) and selenium rectifiers (selenium in contact with aluminium) were produced in about 1925. Important work explaining metal-semiconductor rectifiers was done in the 1930s. In 1948 the transistor was invented by Shockley, Bardeen and Brattain at Bell Laboratories. Then, in the 1960s, an insulating layer of oxide was at last successfully introduced between the metal and the semiconductor to produce what became known as the metal-oxide-semiconductor field-effect transistor, or MOSFET for short. This small and simple device can operate at higher switching speeds and lower currents than bipolar transistors; as the active



Crystal set with cat's whisker (spiral wire).

component of many integrated circuits, it provided the foundation for the 'computational plenty' that we now take for granted. This historical sweep certainly enlivens the section on unipolar devices, and the section on photonic devices is made similarly interesting with the recent social changes that have been brought about by solar power and lasers.

Sze provides a two-page introduction to each section. More intriguing is his short opening preamble in which he shows diagrammatically the relation between the principles of semiconductor devices, not only past and present, but

also for devices not yet realized. The bipolar transistor and MOSFET are seen to evolve through somewhat fantastic forms that may or may not work as devices. This is a small and imaginative addition to a weighty, informative reference volume. The book will be useful not only to technologists searching for their roots, but also to historians looking for the gritty realities of the semiconductor revolution. □

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## Predictable plots

John Lawton

**A Critique for Ecology.** By R. H. Peters. Cambridge University Press: 1991. Pp. 366. £45, \$79.50 (hbk); £17.95, \$29.95 (pbk).

CONSIDER the following propositions. The essence of science is prediction; the best way to make predictions is to regress variables one against the other; the more extravagant goals of science, for instance, that it should seek to understand what is going on, are unnecessary; ecology does not often make predictions; therefore most of contemporary ecology is not science. Briefly, that summarizes the central theme of this extraordinary book. In the author's own words: "Many ecologists . . . have elected to build explanatory 'theories' which stress unity, reality, cause, and mechanism, but jettison predictive power. However, because a construct without predictive power tells us nothing, this choice represents a pathology whereby the trappings of theory beguile us away from predictive power." For predictive power, read graph.

It is a disappointing, uninspiring, negative book. That said, let me first add some qualifying comments, and say some positive things about it. Lest I be accused of simply defending my patch against devastating criticism, the work I have done comes in for relatively little adverse comment. Throughout my career, I have also done what Peters strongly advocates, namely mined the literature for data, and sought to establish (by drawing graphs) patterns in nature. (The difference is that I see the relationships as the beginning of understanding; for Peters they are an end in themselves.) I also share Peters' concern for the lack of apparent progress in solving some long-running problems in ecology, and have written at length ab-

out these. We ought to be soul-mates. Instead, I find myself, more often than not, in fundamental disagreement with him.

Reading the book was like reading an essay written by a dreadfully earnest, but ill-informed, poorly read undergraduate, an essay needing copious red ink on every paragraph. A comprehensive response would require another book, so what follows is only a sample of my concerns.

Peters liberally criticizes several key ideas — for example, density-dependent versus density-independent 'regulation', stability and chaotic dynamics. They are variously dismissed as vague, undefined or useless 'mathemastistry'. In fact he reveals several times that he does not understand these and related concepts and processes, or the mathematics that underpins them. I find it hard to believe that the book was properly refereed.

Analysis of causal links is dismissed as an "infinite research program" and a "singularly ineffective tool". What matters in solving pressing environmental problems are more empirical graphs. The absurdity of this proposition is made clear by two large environmental problems, one solved, the other on its way to being solved. The collapse of peregrine falcon populations throughout the world was reversed only by banning the pesticide DDT (dichlorodiphenyltrichloroethane) and other chlorinated hydrocarbons, and the case for doing so drew on brilliant environmental detective work that laid sufficiently bare the tortuous causal links between pesticides, eggshell thinning and the decline in peregrine numbers to convince politicians to act. Peters would have none of it. Or take the problem of acid deposition and forest decline in Europe. Here, the causal links are many, poorly understood and contentious, but they are becoming clearer. Peters is naive if he believes that graphs of depositions of NO<sub>x</sub> and SO<sub>x</sub> against tree deaths will, on their own, convince politicians that we must drastically curb emissions of these pollutants.

Before they act, politicians want mechanisms, because politicians are not stupid; indeed, they have been taught that although there is a good correlation between the population of storks in the Netherlands and the local birth rate, shooting storks is unlikely to slow human population growth. Understanding causality is not a luxury for effective environmental management, at least not in the world I live in.

Nowhere is the problem of inferring relationships from crude empiricism more clearly illustrated than by the problem of population densities for animals of different body sizes. Peters has made some useful empirical contributions to this field, and the graph relating body size to population density is referred to throughout the book as one of the most useful "predictive theories" (his words, not mine) in ecology. He admits that some species, particularly birds, do not conform well to the overall relationship, but fails to admit that the graph may hide more than it reveals.

For instance, because the graph is based on data drawn entirely from the literature, it under-represents rare species. Also, he explains the slope of the observed relationship (roughly  $-0.75$  on a log-log plot) via per capita resource use and metabolic rates. But this presupposes that all populations are energy-limited, and that equal amounts of energy are available for species of all body sizes. Neither seems likely.

In fact the relationship is poorly understood, and could be generated by several different mechanisms. The very thought is anathema to Peters; what matters is the graph and its 'predictive power'. But poorly understood relationships, based on possibly biased data, are not a wise basis for predicting anything. I prefer to understand what I am doing.

Most areas of science have good and bad parts, skilled and boring practitioners, useless and useful developments. Ecology is no different, and I suspect no worse than any other discipline (despite some contrived analyses by Peters to show otherwise). *A Critique for Ecology* paints a picture of a world that seems vaguely familiar, with which I can agree in part. On closer inspection it reveals a dismayingly patchy grasp of the subject, ruled by a rigid, not to say arcane, view of the philosophy of science, and adherence to the notion that real science is entirely about regressing one variable against another. If that were really the case, editions of *Nature* ought to consist of little more than a compilation of graphs. □

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# Medium-range structural order in covalent amorphous solids

S. R. Elliott

Despite their lack of long-range translational and orientational order, covalent amorphous solids can exhibit structural order over both short and medium length scales, the latter reaching to 20 Å or so. Medium-range order is difficult to measure experimentally and to interpret unambiguously, but a variety of techniques have allowed several types of characteristic structural ordering to be identified and their origin elucidated.

How disordered is the atomic structure of an amorphous solid? Although it is indisputable that such structures are devoid of long-range translational periodicity, the question of the degree of residual structural order present in a noncrystalline material at shorter length scales remains controversial.

Disorder in materials can be manifested in many ways: examples are vibrational, spin and orientational disorder (all referred to a periodic lattice) and topological disorder. I will concentrate principally on the latter, which is the type of disorder associated with the structure of glassy and amorphous solids in which the structure cannot be defined in terms of a periodic lattice.

Determination of the atomic structure of amorphous materials is unfortunately a non-trivial task. Because the structure can be defined essentially only in terms of a 'unit cell' containing an infinitely large number of atoms (as there is no long-range periodic symmetry), a statistical description is unavoidable. The structure of a particular amorphous solid can therefore never be determined unambiguously, and this uncertainty is compounded by the fact that the structure of a noncrystalline material, at both microscopic and macroscopic levels, often depends on details of the method of preparation. Furthermore, in general, more than one experimental structural probe must be used to obtain as full a picture as possible of the structural arrangement in an amorphous solid (see Box 1).

In discussing the structure of amorphous materials, it is useful to consider the types of structural order that can exist in such materials at various length scales. Such a categorization is convenient in two regards: the classification is hierarchical, so that a particular type of order at one length scale can be dictated by order at a smaller scale (but not necessarily the converse);

and the various experimental probes are generally sensitive to structural correlations at different length scales. For reasons of definiteness and brevity, I will consider only covalently bonded amorphous solids, for it is in these materials that the degree and extent of medium-range order is maximized because of the stereochemical (directed) bonding.

## Classification of structural order

We can consider three contiguous length scales<sup>1</sup>: short-range order (SRO) in the range 2–5 Å; medium-range order (MRO) in the range 5–20 Å; and long-range structure (LRS) at distances  $\geq 20$  Å (by definition there is no long-range order, in the form of translational periodicity, in the structure of a glass).

In the case of covalent materials, where directed bonding is dominant, SRO can be characterized in terms of well-defined coordination polyhedra (see Fig. 1*a*). The parameters needed to describe topological SRO are the number,  $N_j$  of nearest neighbours,  $j$ , around an origin atom of type  $i$ , the nearest-neighbour bond-length  $R_{ij}$ , the bond angle  $\theta_{jik}$  subtended at atom  $i$  (where atom type  $k$  may be different from  $j$ ), and the corresponding quantities when atom  $j$  is regarded as the origin, thereby defining the connectivity of the polyhedra.

In addition, the chemical SRO needs to be considered when different types of atoms constitute the coordination polyhedron centred on a particular atom. For nonstoichiometric compositions, excess atoms are accommodated by the formation of 'wrong' (for example, homopolar) bonds, in the absence of coordination or valence changes, and the chemical order that might otherwise occur at the stoichiometric composition is thereby broken; the relevant parameter here is then the fraction of wrong bonds per coordination polyhedron. (Mössbauer

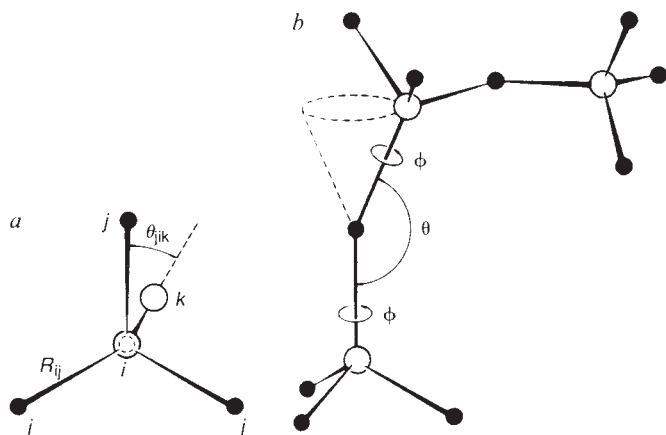


FIG. 1 Illustrations of short-range order (SRO) and medium-range order (MRO). *a*, SRO defined in terms of coordination polyhedra (two- and three-body correlations). *b*, Near MRO defined in terms of connection of polyhedra. The definition of the dihedral angle ( $\phi$ ) is shown (four-body correlation).

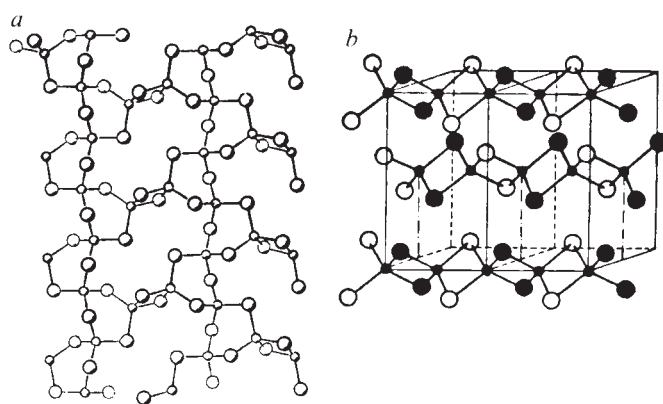


FIG. 2 Structures of crystalline  $\text{GeX}_2$  and  $\text{SiX}_2$  materials (X is S, Se). *a*,  $\text{GeS}_2/\text{GeSe}_2$ , showing 25% proportion of edge-shared tetrahedra resulting in a sheet-like structure. *b*,  $\text{SiS}_2/\text{SiSe}_2$ , showing 100% edge-shared tetrahedra, resulting in a chain-like structure.

spectroscopy can be sensitive to such broken chemical order in certain circumstances<sup>2</sup>.) A related situation is when the different atomic species in the coordination shell around a given origin atom are in fact the same element, but each ligand can have a different charge state, bonding connectivity and so on. An example is the case of 'non-bridging oxygen' atoms (singly bonded, singly negatively charged) introduced into oxide glasses by the incorporation of alkali modifier atoms; for silicates, such configurations are denoted as  $Q_n$  ('quaternary') species, where  $n$  ( $=0-4$ ) is the number of bridging (doubly coordinated) oxygens in the coordination polyhedron (tetrahedron) centred on a silicon atom.

The definition of MRO is more contentious<sup>1,3-5</sup>, but it can simply be regarded as the next highest level of structural organization beyond SRO. In practice, it is helpful to subdivide the types of MRO into three categories<sup>1</sup>, corresponding to increasing length scales. At the shortest length scale (say  $\leq 5$  Å), near-MRO (NMRO) describes the type of connection between the coordination polyhedra. Thus, corner-, edge- or face-sharing of polyhedra leads to very different ordering arrangements on a local scale. A parameter that can be used as a description of the orientational correlations associated with such connectivity is the 'dihedral' (or torsion) angle,  $\phi$ , defined as the angle of rotation about a common bond required to bring into coincidence the projections, on the plane perpendicular to this bond, of the other bonds in the connected units (see Fig. 1b). Thus, NMRO is characterized by four-body correlations. Departures from a uniform (that is, random) distribution of  $\phi$  are correspondingly a hallmark of MRO.

At the next length scale (say  $\sim 5-8$  Å), intermediate MRO (IMRO) can be associated with correlations (phase relationships) between pairs of preferred dihedral angles for neighbouring bonds—that is, triplet correlations between connected polyhedra or, in other words, five-body correlations. Thus, a pronounced degree of IMRO is associated with coordination polyhedra connected together in well-defined relative orienta-

tions. This circumstance leads naturally to the occurrence of 'superstructural' units<sup>1</sup>, which are aggregates of basic polyhedra connected together to form regular rings, or three-dimensional clusters (see Fig. 1c), which can be regarded as constituting alternative building blocks of the structure if they exist in a significantly higher proportion than would be expected on a random statistical basis.

Finally, on a yet larger length scale (say  $8-20$  Å), far-MRO (FMRO) can be associated with the local dimensionality of the covalently bonded amorphous network; this can be ascertained by finding the dimension traced out locally (over distances  $\leq 20$  Å) by a process of bond percolation among the covalent bonds of the structure, neglecting the much weaker Van der Waals bonds<sup>6</sup>. A local dimensionality of 3 would correspond to structural isotropy; local dimensionalities of 2 (layer-like), 1 (chain-like) or 0 (isolated cluster) can arise either from the type of connection between structural units (for example, one-dimensional chain-like structures resulting from edge-sharing of tetrahedra) or from network depolymerization associated with the formation of non-bridging ligands caused by the introduction of network-modifying cations into the structure.

Finally, at the largest length scale, LRS is associated with inhomogeneities in the structure, such as voids and columnar growth morphology in films. Here I will not discuss such large-scale structural imperfections further, concentrating instead on medium-range order.

### Polyhedral connections

Appreciable variations in the type of connections between coordination polyhedra are found in the good glass-forming system,  $AX_2$  ( $A$  is Si or Ge;  $X$  is O, S or Se), where the coordination polyhedra are  $AX_4$  tetrahedra<sup>1</sup>. For the oxides,  $SiO_2$  and  $GeO_2$ , connection is by corner-sharing of tetrahedra through the O atoms; this results in the formation of three-dimensional structures with (relatively) little MRO. But the structures of the chemically analogous glasses, germanium and

#### BOX 1 Experimental structural probes

Several experimental techniques may be used to investigate the structure of amorphous solids, and specifically the MRO. Neutron (or X-ray) diffraction is the obvious choice, but it suffers from the disadvantage that for peaks other than the first (and perhaps the second) in the real-space radial distribution function, obtained by Fourier transformation of the scattering data, unambiguous interpretation in terms of  $n$ -neighbour correlations is not possible because of the overlap of higher-order peaks<sup>1</sup>. This problem is exacerbated for materials containing more than a single type of atom, if just one diffraction experiment is performed, as 'partial' structural information (the structural environment around a particular type of atom) cannot then be obtained. It may be possible to obtain partial structural information by using isotope substitution, if suitable isotopes exist with sufficiently different neutron scattering lengths, or anomalous scattering, if the X-ray photon energy can be tuned to lie either side of an X-ray absorption edge. In fact, diffraction data can only really provide information relating to MRO if it is compared with the calculated scattering characteristics of a structural model for which the atomic positions, and hence the MRO, are known. Nevertheless, the 'first sharp diffraction peak' observed in the structure factor has been regarded as a signature of MRO; a discussion of the structural origin of this peak is given in Box 2.

Other techniques can provide complementary information on MRO in glasses. These techniques should ideally be atom-specific, so that the structural environment of a particular type of atom can be probed, and they should also be sensitive to local conformation, that is, they should probe three-body (or higher) correlations, instead of the two-body correlations probed by conventional single-scattering diffraction experiments. One such method is nuclear magnetic resonance, which, when coupled with the use of 'magic-angle spinning' to reduce the effect of spectral broadening interactions operative in the solid state, can produce high-resolution spectra<sup>1</sup>. The method has the drawback that relatively few nuclei are suitable for study by NMR, and site disorder can cause such a degree of inhomogeneous broadening of resonance lines that a structural

interpretation becomes impossible. Nevertheless, magic-angle spinning NMR is a powerful probe of certain aspects of MRO, such as clusters, where the degree of local order is such that structurally inequivalent sites can be distinguished because their resonance lines have resolvable different chemical shifts. Although most NMR spectra are interpreted by 'finger-printing', where the spectrum of the amorphous solid is compared with those of known, related structures (for example, spectra of molecular species in crystals or solution) to ascertain whether particular structural moieties are present, nevertheless quantitative (pair correlation) structural information can be obtained in principle from NMR experiments by making use of internuclear dipolar interactions whose strength varies as  $r^{-3}$ , where  $r$  is the interatomic spacing.

Vibrational spectroscopy, and in particular Raman scattering, is another non-diffraction structural probe which can be especially sensitive to the presence of MRO. Highly ordered regions of an amorphous structure, associated with a considerable degree of MRO, can support vibrational excitations which, when 'vibrationally decoupled' from the rest of the network, may produce intense, narrow Raman lines. Such signatures of MRO can be even more apparent if the vibrational mode of a superstructural unit is a symmetric breathing motion, as this will result in the associated Raman line being strongly polarized, (that is, the band measured in the HH configuration is considerably more intense than that measured in the HV configuration, where HH and HV refer to the polarization directions in the incident and scattered beams (H, horizontal; V, vertical)). Such symmetric modes can also lead to an enhanced matrix element for scattering; that is, stronger coupling constants relating the induced polarizability and the displacement amplitudes. As a result, Raman scattering is an extremely sensitive probe for the existence of regular superstructural units in the structure of amorphous solids, but, correspondingly, it is not a quantitative technique: the intensities of peaks in the HH Raman spectrum associated with MRO are generally greatly enhanced with respect to the integrated area of the corresponding feature in the true vibrational density of states.



silicon chalcogenides, seem to be intriguingly different. The low-pressure/low-temperature crystalline form of  $\text{GeS}_2$  and  $\text{GeSe}_2$  consists of 50% of the tetrahedra with edge-shared connections; these act as bridges for chains of corner-shared tetrahedra, thereby resulting in a sheet-like two-dimensional structure (Fig. 2a). In the low-pressure/low-temperature polymorph of crystalline  $\text{SiS}_2$  and  $\text{SiSe}_2$ , all tetrahedra have edge-shared connections; this configuration results in a chain-like one-dimensional structure (Fig. 2b). There has been considerable controversy over the structures of glasses of these materials. Of particular concern is the extent of the similarity between crystalline and non-crystalline structures, as indicated by the proportion of edge-shared tetrahedra (that is, the NMRO<sup>7,8</sup>).

In the case of g- $\text{GeSe}_2$ , high-resolution neutron diffraction<sup>9</sup> has provided clear evidence for anomalously short Ge-Ge distances in the structure, associated with the four-membered ( $\text{Ge}_2\text{Se}_2$ ) rings characteristic of edge-shared connections between tetrahedral units (see Fig. 2). The fraction of edge-sharing tetrahedra found in the glass structure was  $\sim 40\%$  (as compared with 50% for the crystal); a molecular-dynamics simulation<sup>10</sup> of the structure of g- $\text{GeSe}_2$ , using three-body interaction potentials to mimic covalent forces, predicts  $\sim 32\%$  edge-sharing tetrahedra, with a radial distribution function and structure factor in generally good agreement with the neutron data<sup>9</sup>.

Neutron diffraction data also exist for g- $\text{SiSe}_2$  (refs 11, 12), and a systematic modelling study of the structure of this material, using serial addition of atoms, has been made in an attempt to fit the scattering data<sup>13,14</sup>. As in the structure of the crystalline polymorph, the dominant structural motif in the glass is found to be the edge-shared tetrahedron. The configuration of  $\text{SiX}_4$  tetrahedra can be represented as  $E_n$  species, where E stands for edge-sharing, and  $n$  ( $=0-2$ ) is the number of edge-shared connections per tetrahedral unit. Modelling studies<sup>13,14</sup> found, however, that not all tetrahedra had  $E_2$  configurations; this implies that the structure of the glass is not composed entirely of random chains of linked tetrahedra, as originally suggested on the basis of a comparison of the Raman scattering data for the glass with that for the crystal<sup>15,16</sup>. A later interpretation<sup>17</sup> of the Raman spectra of g- $\text{Si}_x\text{Se}_{1-x}$  led to the suggestion that some  $E_1$  and  $E_0$  tetrahedral configurations were also present. Further confirmation for the simultaneous presence of  $E_2$ ,  $E_1$  and  $E_0$  configurations in the structure of  $\text{SiX}_2$  glasses (where X is S, Se) has come from <sup>29</sup>Si magic-angle-spinning nuclear magnetic resonance (NMR)<sup>18,19</sup> (see Box 1); the relative proportions of such tetrahedral configurations have been estimated<sup>18</sup> from the NMR data to be 25%  $E_0$ , 50%  $E_1$  and 25%  $E_2$ . The corner-sharing bonds associated with such  $E_0$  and  $E_1$  units cause a cross-linking of the chains, and it has been postulated<sup>17</sup> that 'cross-linked chain clusters' are formed—that is, rings containing chain-like fragments consisting of four  $E_1$  and two  $E_0$  units (see Fig. 3). A modelling study<sup>13,14</sup> demonstrated that the neutron diffraction data<sup>11,12</sup> are best fitted by a mixture of structural groupings (Fig. 3), including  $\sim 15\%$  of cross-linked chain clusters, with the rest of the structure in the form of random chains of linked tetrahedra, each containing an average of seven  $E_2$  units. In  $\sim 15\%$  of these random chains, two chains run locally parallel over a correlation length corresponding to two or three  $E_2$  units. This is perhaps one of the most comprehensive pictures we have yet of the type and extent of MRO in the structure of an inorganic amorphous solid.

The Raman spectra of  $\text{AX}_2$ -like glasses are generally rich<sup>20</sup>, with many narrow lines, surprising in a noncrystalline solid where the effect of structural disorder is generally to broaden Raman lines substantially in comparison with those characteristic of crystals. There are two reasons for this broadening: the absence of periodicity means that, in glasses, extended vibrational excitations ('phonons') cannot be described in terms of  $k$ -states, with the consequence that selection rules associated with optical transitions are relaxed and all regions of the vibrational density of states are optically accessible; furthermore, the

structural disorder characteristic of noncrystalline materials generally broadens features in the vibrational density of states. Nevertheless, in chalcogenide glasses in general, and  $\text{AX}_2$ -like materials in particular, vibrational spectra (for example, Raman spectra) have sharp ('molecular-like') peaks as a result of vibrational decoupling<sup>21</sup> of modes associated with cation-centred structural units (such as  $\text{AX}_4$ -like tetrahedra) because the bridging chalcogen atoms have bond angles near  $90^\circ$ .

One particular narrow peak in the Raman spectrum of  $\text{AX}_2$ -type glasses has long been associated with MRO because of its anomalously strong dependence<sup>22</sup> on composition: it was

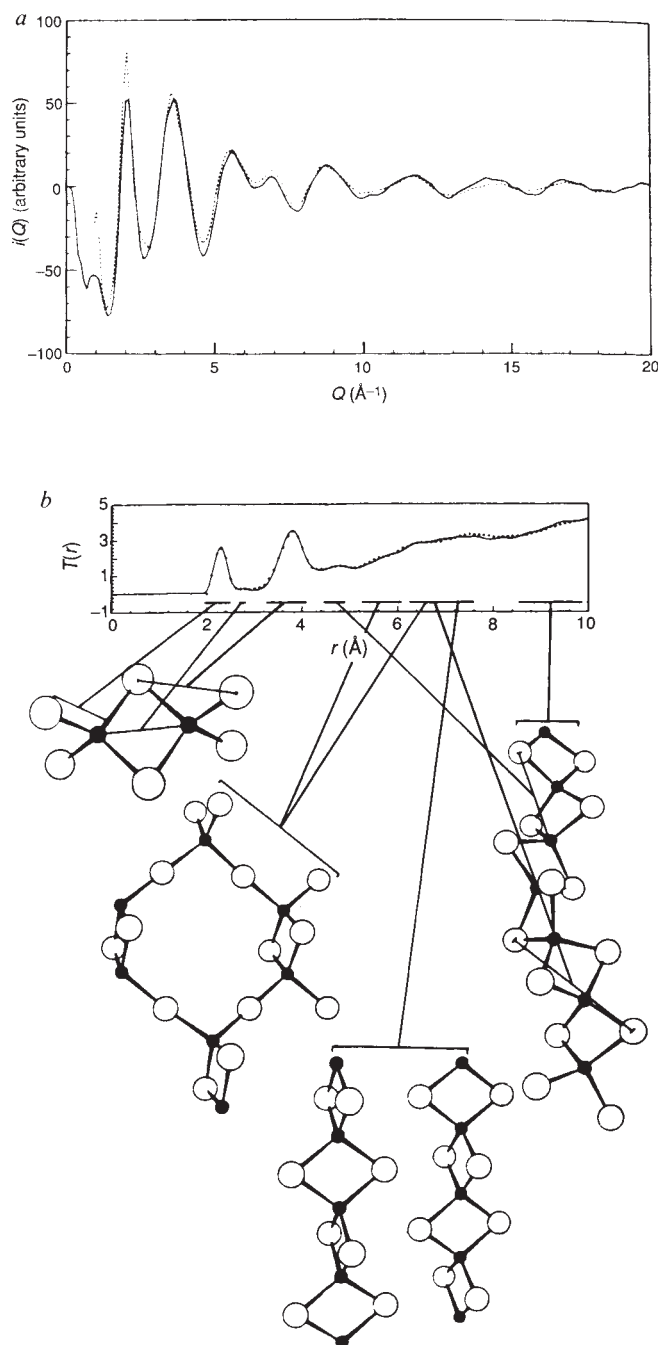


FIG. 3 Experimental neutron diffraction data<sup>11,12</sup> for g- $\text{SiSe}_2$  compared with the results of a structural model<sup>13,14</sup>. a, Neutron-scattering intensity function (dashed line, experiment; solid line, model). b, Radial distribution function (dashed line, experiment; solid line, model). Also shown are the contributions to designated regions of the overall radial distribution function of two-body correlations associated with different structural groupings.

assumed to be a vibrational mode associated with larger-scale structural features, characteristic of MRO, that are broken up preferentially when the composition changes. In the case of g-GeSe<sub>2</sub>, this anomalous Raman peak occurs as a small, strongly polarized peak at  $\sim 220\text{ cm}^{-1}$  (26.5 meV) associated with the prominent A<sub>1</sub> symmetric-stretch 'breathing' mode of GeSe<sub>4</sub> tetrahedra which lies at  $\sim 200\text{ cm}^{-1}$  (25 meV). It has therefore been termed<sup>23</sup> the 'companion line'. The structural origin of this companion line has long been controversial. Originally, it was ascribed to vibrations of large regular rings<sup>22</sup>, and later<sup>23</sup> to the vibrations of wrongly bonded Se-Se 'dimers' postulated to lie at the edges of 'rafts', a term referring to layer-like moieties of the crystalline structure (see Fig. 2a). Such rafts are too ordered, however, to be consistent with the diffraction data for the glass<sup>8</sup>. The present consensus of opinion<sup>20,24</sup> seems to be that the companion Raman line in AX<sub>2</sub>-like glasses is simply associated with vibrations of the four-membered (A<sub>2</sub>X<sub>2</sub>) rings characteristic of edge-shared connections between tetrahedra. Presumably, this type of connection is particularly sensitive to rupture for departures of the composition from the stoichiometric composition AX<sub>2</sub>.

## Rings

Rings of atoms in a covalently bonded amorphous network only become a feature of MRO if they exist either in a proportion greater than expected for an 'ideal' continuous random network of the material (that is, they are 'superstructural' units) or if they are more regular than would otherwise be expected (that is, there is a pronounced well-defined correlation between neighbouring dihedral angles). I have already mentioned one type of ring structure that is characteristic of MRO, the four-membered A<sub>2</sub>X<sub>2</sub> rings associated with edge-sharing tetrahedra in AX<sub>2</sub> glasses. These rings will not be discussed further in this section, however, attention being paid to larger rings.

Perhaps the most celebrated example of a ring acting as a superstructural unit in the structure of a glass is the 'boroxol' ring found in g-B<sub>2</sub>O<sub>3</sub>. This is a regular, planar, six-membered B<sub>3</sub>O<sub>3</sub> ring, composed of three corner-sharing planar BO<sub>3</sub> triangles (the basic structural unit in B<sub>2</sub>O<sub>3</sub>) joined together in a triangular arrangement through their apices (see Fig. 4a). The most striking experimental manifestation of the boroxol ring is in the Raman scattering spectrum (Fig. 4a): a highly polarized and very narrow peak is observed at  $808\text{ cm}^{-1}$ , indicative of a

symmetrical stretching vibrational mode which is localized because of appreciable vibrational decoupling of the mode from other extended (phonon-like) vibrational modes associated with the framework structure. There is no change in the  $808\text{ cm}^{-1}$  Raman line on substituting a different B isotope, but there is a pronounced shift in frequency when the O isotope is changed<sup>25</sup>. This implies that only O motion is involved in the vibrational mode responsible for the  $808\text{ cm}^{-1}$  Raman peak, and the observed 1:3:3:1 intensity ratio for the resolved quadruplet line when a 50:50 mixture of <sup>16</sup>O and <sup>18</sup>O isotopes is present is consistent with a random isotopic substitution in a structural unit containing just three O atoms, such as the boroxol ring<sup>25</sup>. It is well known<sup>1</sup> that matrix-element enhancement of symmetric vibrational modes can occur in Raman spectra, making Raman scattering a particularly powerful probe of MRO in the form of regular rings, but it may not be a quantitative probe for structural features that are associated with MRO and are responsible for a particular vibrational mode (see Box 1). This ambiguity has led to considerable controversy<sup>26</sup> over the proportion of boroxol rings in the structure of g-B<sub>2</sub>O<sub>3</sub>. Estimates for the fraction, *f*, of B atoms in boroxol rings have been inferred from neutron diffraction data<sup>27</sup> (*f* = 0.6) and from <sup>11</sup>B NMR experiments<sup>28</sup> (*f* = 0.8); for comparison, a value of *f* = 0.75 corresponds to a network containing equal numbers of boroxol rings and independent BO<sub>3</sub> triangles. But recent inelastic neutron scattering experiments<sup>29</sup> of g-B<sub>2</sub>O<sub>3</sub> (where the coupling constants relating the scattering intensity to the vibrational displacement amplitudes are not enhanced anomalously for the case of symmetric modes) have shown that only a very small peak is seen in the vibrational density of states at  $808\text{ cm}^{-1}$ , indicating perhaps that the concentration of boroxol rings in this material is lower than originally believed.

Similar, but less intense, narrow polarized Raman lines have been observed in other oxide glasses. For example in g-SiO<sub>2</sub>, two such lines at  $495\text{ cm}^{-1}$  (D<sub>1</sub>) and  $606\text{ cm}^{-1}$  (D<sub>2</sub>) are clearly apparent in the Raman spectrum<sup>30</sup>. The structural origin of these Raman bands has been contentious but, on the basis of isotopic substitution experiments, the D<sub>2</sub> line has been ascribed to the symmetric breathing mode involving O atoms in a planar six-membered (Si<sub>3</sub>O<sub>3</sub>) ring (topologically similar to the boroxol ring), and the D<sub>1</sub> line has been ascribed more tentatively to the same motion in a regular, but slightly puckered, eight-membered ring. The O motion in such rings is vibrationally decoupled from the rest of the network if the ratio of the bond-bending and bond-stretching force constants satisfies a particular analytical relationship involving the angles subtended within the rings at the Si and O atoms<sup>31</sup>. Actual numerical calculations of the vibrational density of states for a continuous random network (CRN) of g-SiO<sub>2</sub> containing six- and eightfold rings have demonstrated, however, that narrow vibrational bands are only obtained if the rings are extremely regular<sup>32</sup>. The structural origin of these Raman features therefore remains in some doubt.

Other noncrystalline systems where rings are believed to play an important part in the structure are the elemental chalcogens, amorphous sulphur and selenium. In the case of sulphur, for example, the various crystalline polymorphs are composed of packings of discrete cyclo-octasulphur (S<sub>8</sub>) rings. On melting, the integrity of the rings is preserved until the well-known λ-transition occurs (*T*<sub>λ</sub> = 433 K), where the rings rupture to form long polymeric chains, thereby causing the viscosity to increase markedly. There has been a long-standing debate about the structure of sulphur (and selenium) glasses quenched from the melt with respect to the relative proportions of rings and chains. Neutron scattering<sup>33</sup> indicates that the structures of molten sulphur and sulphur glass are similar, and significantly different from the structure of a noncrystalline aggregate of S<sub>8</sub> rings predicted by a molecular-dynamics simulation,<sup>34</sup> indicating that chains are the dominant structural motif. The same situation seems to prevail in g-Se.

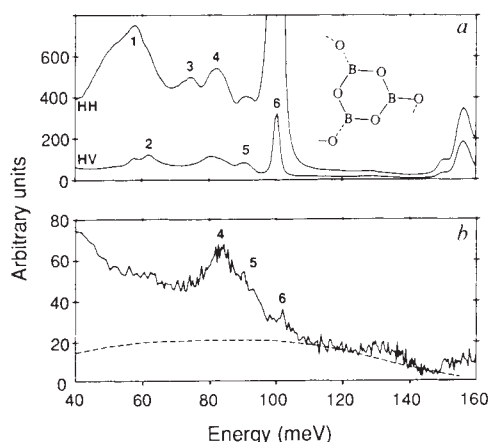


FIG. 4 Vibrational spectroscopy of g-B<sub>2</sub>O<sub>3</sub>. a, Raman spectra<sup>25</sup> showing the highly polarized, narrow band at 100 meV ( $808\text{ cm}^{-1}$ ) ascribed to the symmetric-stretch breathing mode of the oxygen atoms in a boroxol ring (see inset). HV and HH refer to different polarization configurations of the incident and scattered light (H, horizontal; V, vertical). b, Inelastic neutron scattering spectrum<sup>29</sup>, indicating the absence of a significant feature in the vibrational density of states at 100 meV ( $808\text{ cm}^{-1}$ ).



## Clusters

Well-defined clusters of atoms can be regarded as being orientationally ordered arrangements of regular rings and, as such, they represent the next stage in the hierarchy of MRO. As for the case of rings themselves, clusters may be discrete (that is, unconnected by covalent bonds to the rest of the network), in which case the structure consists of a packing of such clusters, or they may be fully incorporated into the matrix of the glass, thereby contributing local regions of high MRO. Several covalently bonded amorphous solids have cluster-like MRO.

One example is the elemental pnictogen, amorphous red phosphorus, made by heat-treating white phosphorus. This material produces a very pronounced first sharp diffraction peak (see Box 2) in the structure factor<sup>35</sup>, often taken to be a signature of appreciable MRO, and the Raman spectrum<sup>36</sup> is remarkably structured for an amorphous solid, the most prominent band at  $\sim 350\text{ cm}^{-1}$  being strongly polarized, indicative of a symmetric vibrational mode (see Fig. 5a). The Raman spectrum of the monoclinic 'Hittorf' crystalline polymorph of phosphorus is remarkably similar to that observed in the amorphous solid (see Fig. 5a), and calculations of the vibrational characteristics of the cage-like  $P_8$  and  $P_9$  clusters found in the Hittorf form demonstrate that symmetric breathing modes of these clusters are responsible for the sharp, polarized features observed in the Raman spectrum of the amorphous material<sup>36</sup>. In the crystalline phase, the  $P_8$  and  $P_9$  clusters are connected together to form

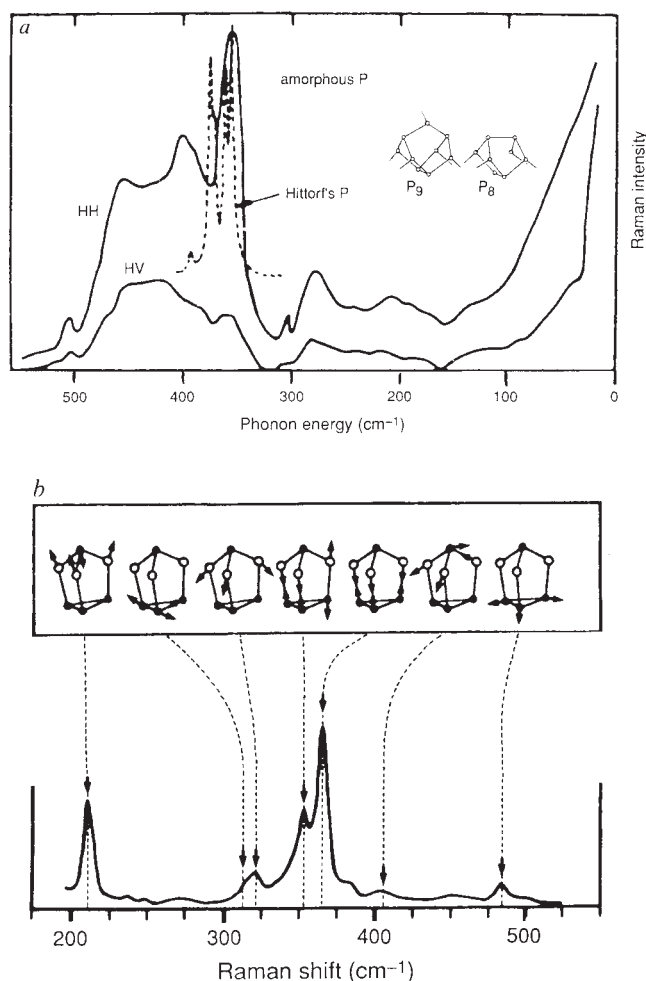


FIG. 5 Cluster-like MRO in amorphous solids. *a*, Raman spectra<sup>36</sup> of a-P, with, inset, the  $P_8$  and  $P_9$  clusters (characteristic of the monoclinic 'Hittorf' crystalline polymorph), the vibrational modes of which are believed to be responsible for the sharp peaks in the spectrum. *b*, Raman spectrum<sup>48</sup> of g- $P_2Se_3$ . Peaks are assigned in terms of the vibrational modes of a  $P_4Se_3$  molecule (inset).

extended 'tubes' with a pentagonal cross-section (see inset to Fig. 5a). In the amorphous phase, however, it is very unlikely that this type of order persists; it is more probable that isolated  $P_8$  or  $P_9$  clusters occur, embedded in and covalently bonded to a matrix composed of a threefold coordinated continuous random network<sup>37</sup>.

Discrete cluster-like molecular species have been identified in some amorphous chalcogenide systems. In the case of as-deposited, evaporated amorphous thin films of arsenic sulphide, diffraction data can be interpreted satisfactorily in terms of a packing of almost spherical  $As_4S_4$  molecules (weakly bonded together by van der Waals interactions), with minimal cross-linking between (ruptured) molecular species<sup>38,39</sup>. Such a structure is, perhaps, not too surprising, as the stable vapour species above a melt of  $As_2S_3$  are  $As_4S_4$  and  $S_2$  molecules.

Perhaps the most interesting of all amorphous chalcogenide materials in relation to MRO is the P-Se system, because of its propensity to form  $P_4Se_n$  ( $n=3-10$ ) cage-like molecular clusters<sup>40</sup>. In Se-rich P-Se glasses, neutron diffraction and extended X-ray absorption fine structure measurements<sup>40</sup>, as well as magic-angle spinning NMR spectra<sup>19,41</sup>, provide evidence for some  $P_4Se_n$  clusters (mainly with  $n=10$  for high Se contents) embedded in an amorphous Se matrix. The  $P_4Se_{10}$  molecule has four terminator  $P=Se$  double bonds oriented in a tetrahedral manner; in the melt-quenched glass, it is probable that these double bonds break, with the result that the four terminating Se atoms can form covalent cross-links to the rest of the structure. Recent  $^{31}P$  spin-echo and magic-angle NMR experiments<sup>42</sup> on Se-rich P-Se glasses, however, indicate that clustering may be less prevalent than previously believed.

The more interesting glass is  $P_2Se$ , which has also been studied using neutron diffraction<sup>44,45</sup>, extended X-ray absorption fine structure<sup>46</sup> and NMR<sup>41,43</sup>. Here, there is overwhelming evidence that the structure consists almost entirely of discrete quasi-spherical  $P_4Se_3$  cage-like molecules (see inset to Fig. 5b), with a small excess of P. For example, the neutron static structure factor,  $S(Q)$  (see Box 2) for g- $P_2Se$  can be fitted almost exactly for  $Q \geq 6\text{ \AA}^{-1}$  by the form factor calculated for the  $P_4Se_3$  molecule<sup>44,45</sup>. Inelastic neutron scattering measurements<sup>47</sup> reveal a highly structured vibrational density of states, the peaks in which can be assigned to the vibrational excitations of a  $P_4Se_3$  molecule. Raman spectra<sup>48</sup> support this assignment, and the higher resolution of Raman scattering compared with inelastic neutron scattering allows more of the vibrational modes to be distinguished (Fig. 5b). Finally, quasi-elastic neutron scattering measurements<sup>45</sup> of g- $P_2Se$  have revealed a quasi-elastic broadening of the elastic line at elevated temperatures (300–400 K) (but below the glass-transition temperature,  $T_g = 438\text{ K}$ ), indicating the presence of (diffusive) atomic motion. These observations have been interpreted<sup>45</sup> in terms of a rotational diffusional motion of discrete  $P_4Se_3$  molecules (whose centres of mass are randomly distributed in the glassy structure), similar to that which occurs in the plastic phase ( $T \geq 355\text{ K}$ ) of crystalline  $P_4Se_3$ . Measurements from  $^{31}P$  spin-echo NMR<sup>43</sup> have also provided evidence for orientational mobility of these units. Thus, g- $P_2Se$  may be the first known example of an inorganic 'plastic glass', where topological and dynamic orientational disorder are present simultaneously.

## Future prospects

Much progress has been made recently in our understanding of medium-range order in the structure of glasses, particularly of covalently bonded materials where 'superstructural units' (such as regular rings or clusters) are prevalent. But much remains unclear and ill-understood. It seems that further substantial progress in this area will only come from additional advances in experimental techniques and in theoretical descriptions of the disordered state of matter.

On the experimental side, it is very desirable that new techniques are developed which are more sensitive to conformation



(or orientational order) in disordered materials. One potentially powerful method involves NMR, in which conformational and connectivity information can in principle be obtained from interactions such as  $J$ - $J$  couplings in two-dimensional spectra<sup>49</sup>. Although this approach has often been used to study conformations of organic molecules in the liquid state, typically using two-dimensional homonuclear shift-correlated spectroscopy (COSY), its use in solid-state studies is hampered by the line-broadening interactions (such as dipolar, quadrupolar or chemical-shift anisotropic) which are normally dominant in solids. Nevertheless, line-narrowing techniques, such as magic-angle spinning and certain multiple-pulse sequences, can be used in such cases, and it is conceivable that under favourable circumstances, conformations (of, for instance, rings, chains or clusters) and connectivities of atoms in amorphous solids could be investigated in this way. Recently, <sup>29</sup>Si magic-angle spinning NMR COSY experiments have been used on glasses to investigate the connectivity of Si sites in silicates<sup>49</sup>. Phosphorus-containing glassy solids (such as phosphates or phosphorus chalcogenides) may also be amenable to this approach.

It has been suggested<sup>50</sup> that nonlinear optics could be an experimental probe for local chiral ordering in amorphous covalent solids. For amorphous tetrahedrally coordinated

materials (Si, Ge), chirality has been discussed in terms of the dihedral angle,  $\phi$ , associated with six-membered rings in the structure<sup>50,51</sup>. Perfect 'boat-like' ( $\phi = 0^\circ$ ) and 'chair-like' ( $\phi = 60^\circ$ ) rings both have mirror symmetries, whereas the 'twisted-boat' ring ( $\phi \neq 0^\circ, 60^\circ$ ) has only 2-fold rotation symmetry and no mirror symmetry: twisted rings therefore occur in enantiomorphous pairs, each with a distinguishable 'left-hand' ( $-60^\circ < \phi < 0^\circ$ ) and 'right-hand' ( $0^\circ < \phi < 60^\circ$ ) chirality<sup>50</sup>. Although amorphous materials must be (optically) isotropic overall on a macroscopic scale, in principle domains of different chirality could exist on an intermediate microscopic scale. Examination<sup>51</sup> of an all-even-ring model<sup>52</sup> of a-Si(Ge) has demonstrated that it contains rings mostly of the same handedness. Incoherent three-wave mixing (second-harmonic generation) has been suggested<sup>50</sup> as an experimental probe of such local chirality, at least in the 'inverse electro-optic' limit where two incident waves, having nearly the same (high) frequency, are mixed, and the response at a (low) difference frequency is monitored. Here, the intensity of the scattered radiation should be a direct measure of the size of the chiral coherence lengths. Such experiments have not yet been done on covalent amorphous solids, but it is more likely that chirality would be measurable in this way for stoichiometric chalcogenide or oxide glasses

## BOX 2 The first sharp diffraction peak

A feature of diffraction data of covalently bonded amorphous solids which has long been associated with the presence of MRO is the 'first sharp diffraction peak' (FSDP) occurring at values of scattering vector  $Q = 1\text{--}2 \text{ \AA}^{-1}$ , depending on the material. The structural origin of the FSDP has, however, been extremely controversial.

The FSDP is anomalous in a number of ways. It is considerably narrower than the other peaks in the structure factor,  $S(Q)$ —hence its name. Furthermore, its behaviour as a function of temperature and pressure differs from that of the other peaks in  $S(Q)$ : in chalcogenide glasses, the FSDP increases (reversibly) in intensity with increasing temperature<sup>67</sup>, whereas other peaks in the structure factor decrease in intensity with increasing temperature according to the normal Debye-Waller behaviour; with increasing pressure, it rapidly decreases in intensity, and shifts to higher  $Q$  (refs 68, 69), unlike other peaks in  $S(Q)$ .

Detailed microscopic understanding of the structural origin of the FSDP has remained elusive<sup>1</sup>. For the case of  $AX_2$ -type chalcogenide glasses (where A is Si, Ge; X is O, S, Se, Te), however, it seems that cation-centred correlations (A-A and, to a lesser extent, A-X) are primarily responsible for the FSDP. Such evidence has come from anomalous X-ray scattering experiments<sup>70</sup> on g-GeSe<sub>2</sub>, from isotopic substitution neutron diffraction experiments on (liquid) GeSe<sub>2</sub> (ref. 71) and from molecular-dynamics simulations<sup>10</sup> of the structure of g-GeSe<sub>2</sub>. Finally, although the FSDPs of different chalcogenide and oxide glasses are seen at different scattering vectors,  $Q_1$ , when the structure factors are scaled to the nearest-neighbour bond-length,  $r_1$ , the FSDPs appear rather similar (ref. 72; see Fig. 6).

Proposals made in the past for the MRO structural origin of the FSDP can be divided into two categories: quasi-crystalline structural configurations or clusters.

Because the crystal structures of many glass-forming chalcogenides (for example, As<sub>2</sub>S<sub>3</sub> or GeSe<sub>2</sub>) are layer-like, it was natural to identify the FSDP with a Bragg-like peak in the scattering factor. For example, the (020) Bragg peak of the orpiment crystalline polymorph of As<sub>2</sub>S<sub>3</sub> occurs at a very similar  $Q$  to that at which the FSDP is observed in the glassy phase<sup>73</sup>. Thus, it has been assumed that pseudo-crystalline layer-like structural arrangements with spatial repeat  $d$  are present in the glass<sup>67</sup>; the FSDP position is then given roughly by  $Q_1 \approx 2\pi/d$ . This pseudo-crystalline model is inconsistent, however, with some experimental observations. The FSDP is also produced by some chalcogenide materials in the liquid state<sup>74</sup>; it is extremely unlikely that pseudo-crystalline arrangements having correlation lengths 20–30 Å should survive in the liquid. Furthermore, and perhaps more tellingly, a layer-like interpretation cannot be a general explanation for the occurrence of the FSDP, as the feature is observed even in glasses (such as SiO<sub>2</sub>), where the crystalline polymorphs are not layer-like. Nevertheless, the attraction of an interpretation in terms of parallel ordered configurations remains strong; recent work<sup>75</sup> has shown that structural correlations between pairs of ribbon-like struc-

tural configurations in GeS<sub>2</sub> or GeSe<sub>2</sub> can in certain circumstances give rise to a FSDP<sup>75</sup>.

Another explanation for the FSDP involves correlations between (ill-defined) clusters. It is well known<sup>76</sup> that the structure factors of molecular liquids, such as CCl<sub>4</sub>, also show sharp, well-defined FSDPs (albeit at different values of  $Q$  from those characteristic of inorganic glasses). For such systems,  $S(Q)$  can be regarded as the sum of two distinct terms, one arising from intermolecular correlations and given by an intermolecular interference function,  $D_m(Q)$ , and the other resulting from intramolecular correlations and given by a molecular form factor  $f_m(Q)$ : that is,  $S(Q) = D_m(Q) + f_m(Q)$ . Because intramolecular correlations in molecular liquids are well defined, the oscillations in  $f_m(Q)$  persist to high values of  $Q$  and are the dominant contribution to  $S(Q)$  in this region. Intermolecular correlations are much less well defined, and consequently peaks in  $D_m(Q)$  are rapidly damped with increasing  $Q$ . Either the first, and most prominent, peak in  $D_m(Q)$  forms the first peak in  $S(Q)$ , in other words the FSDP, or else it is an artefact resulting from the addition of a decreasing function ( $f_m(Q)$ ) and an increasing function ( $D_m(Q)$ ). In this picture, the FSDP by itself therefore has minimal structural significance. The role of correlations between clusters in producing the FSDP in chalcogenide, oxide and other glasses has also been stressed<sup>77,78</sup>; in general, however, the structural identity of such clusters in glasses remains obscure.

I have recently proposed a new explanation<sup>79</sup>, in which the FSDP is ascribed to a chemical-order pre-peak (in the concentration-concentration partial structure factor,  $S_{cc}(Q)$ ), in the Bhatia-Thornton formalism<sup>80</sup>), arising from the clustering of interstitial voids around cation-centred 'clusters'; in the case of  $AX_2$ -type glasses, the clusters are taken to be the  $AX_4$  tetrahedra themselves. Blétry<sup>81</sup> has discussed the structure of the tetrahedrally coordinated elemental amorphous semiconductors, a-Si and Ge, in terms of a packing of atoms and atom-sized voids, and he ascribed the first peak in  $S(Q)$  at  $Q \approx 2 \text{ \AA}^{-1}$  (not previously regarded as an FSDP in these cases) to a pre-peak in  $S_{cc}(Q)$  resulting from the chemical ordering voids around the atoms. I believe, however, that clustering of smaller interstitial voids around clusters of atoms in covalently bonded glasses (and liquids) is a general occurrence, and this feature is the reason for the ubiquity of the FSDP in these materials. A recent interstitial-void analysis of models of v-SiO<sub>2</sub>, for example, has shown that such voids are situated at remarkably well-defined distances from Si (but not O) atoms<sup>82</sup>.

This association of the FSDP with correlations involving interstitial voids naturally explains the observed effect of pressure: the application of pressure causes a densification of the glass structure through a diminution of interstitial volume, thereby leading to a decrease in the intensity of the FSDP. The anomalous temperature dependence of the FSDP essentially occurs because, as the temperature of a glass increases, its density decreases. The survival of the FSDP into the molten state can also naturally be understood on this basis.



(such as  $AX_2$  or  $B_2X_3$  systems), where all rings are even (recall, for instance, that the crystalline polymorph of silica, quartz, is chiral), than for the amorphous elements, Ge or Si, where the structure is likely to be a mixture of even and odd rings with negligible net chirality<sup>51</sup>.

Nevertheless, optical activity can, under certain circumstances, probe chirality in glasses<sup>50</sup>. In recent experiments on bulk glassy semiconducting  $As_2S_3$ , both natural and photo-induced optical activities were observed<sup>53</sup>. Moreover, considerable diffuse light scattering was observed after optical excitation of the glass<sup>53</sup>; this behaviour could result from optically-induced changes in local optical activity<sup>50</sup>. Possible local chiral units in the structure of  $As_2S_3$  are helical chains of As and S atoms (cross-linked at the As atoms by bridging S atoms). These are present in the crystal structure, and models<sup>54</sup> indicate that they are present, to a lesser degree, in the structure of glassy  $As_2S_3$ . Although the origin of optically induced optical anisotropy in chalcogenide glasses is not yet understood, it is conceivable that the absorption of polarized light by glassy  $As_2S_3$  causes a 'flipping' of twofold coordinated S atoms in helical sites so that a degree of local helical order is established—that is, domains of local chirality are produced. The possible use of photo-induced optical anisotropy in chalcogenide glasses as a probe of local chirality warrants further study.

There are many gaps in our theoretical understanding of medium-range order in the structure of glasses. Here I have adopted the (geometric) approach that MRO can be regarded as being related in an hierarchical way to short-range order in the case of covalently bonded amorphous materials. This has been termed 'propagated' SRO elsewhere<sup>55</sup>. The viewpoint is useful because, in such materials, the SRO is so well defined, with such strong orientational directionality, that correlations in position and orientation between neighbouring structural units are very likely.

In certain cases, however, the converse may be true: stereochemical SRO may be a consequence of the existence of a well-defined MRO<sup>56</sup>. This idea has been proposed<sup>56,57</sup> specifically for the case of transition metal-metalloid (TMM) glasses, such as  $Pd_{80}Si_{20}$ . In the case of crystalline TMM materials, trigonal prismatic coordination of metalloid atoms can be regarded as arising from the introduction of 'chemical twinning' planes containing the metalloid atoms into close-packed crystalline arrangements of the metal atoms; the composition of the TMM alloy can be varied by changing the periodicity of the chemical twinning planes. In glasses of these materials, it has been suggested<sup>57</sup> that MRO is associated with domains of size 10–20 Å, within each of which there are more-or-less ordered arrays of chemical twinning planes, and in such planes are therefore located the trigonal prismatic units which are believed to characterize the SRO in these amorphous metals; the assumption is that disorder is introduced by the orientation of the twinning planes being different in each domain.

The MRO may also determine the SRO in other (pseudo-) close-packed noncrystalline systems. One example concerns alkali or alkaline earth silicate glasses<sup>56</sup>, where the O anions can be regarded as being effectively nearly close-packed, and (some of) the interstices can be regarded, to a first approximation, as the sites occupied by the alkali or alkaline-earth 'modifier' cations.

A second-difference, isotope-substitution neutron diffraction study of a calcium silicate glass<sup>58</sup>, providing direct evidence for Ca–Ca correlations, has revealed that the Ca ions are not distributed randomly in the silicate matrix, but instead seem to occupy octahedral interstices in the O sublattice (as in the corresponding crystal). Furthermore, these  $CaO_6$  octahedra seem to be connected by edge-shared connections, resulting in sheet-like structures (again as found in the corresponding crystal), and these layers are interconnected by  $SiO_4$  tetrahedra, the Si atoms therefore occupying the tetrahedral interstices in the O packing. Thus, the SRO of the  $Ca^{2+}$  cations can be regarded

as resulting from the MRO associated with the close-packed O sublattice.

For alkali silicate glasses, the picture seems to be similar. Magic-angle spinning NMR has revealed that, at certain stoichiometric compositions, essentially only one type of  $SiO_4$  tetrahedral species exists, as described by the number  $n$ , of bridging oxygens per tetrahedron (denoted  $Q_n$ ,  $n=0-4$ ). For the case of disilicate glass compositions, for example,  $Q_3$  species are dominant<sup>59</sup>: that is, there is only one negatively charged non-bridging O atom per  $SiO_4$  tetrahedron, as in the corresponding crystal. In reality, however, there is some speciation even at stoichiometric compositions, most notably in the lithium silicates<sup>59</sup>. The observation of preferred  $Q_n$  species at certain compositions, instead of a statistical distribution determined by the composition, implies, of course, that the local structural environment (SRO) of the alkali modifier cations is also highly ordered.

A recent attempt<sup>60</sup> to understand the variation with composition of the proportion of different  $Q_n$  species in alkali silicate glasses uses a thermodynamic approach involving disproportionation equilibria between the species; this approach would be valid in the molten state, and the resulting distributions of  $Q_n$  species are assumed to be frozen in on quenching the melt through the glass-transition temperature to form the glass. It was found that the quantity that determines the distribution is the 'bond-ordering energy' arising from the (coulombic) interaction between non-bridging and bridging O sites,  $\Delta E = E_{BB} + E_{NN} - 2E_{NB}$  (where B and N refer to bridging and non-bridging oxygens respectively), rather than the configurational entropy. Thus, the structure of alkali silicate glasses would be

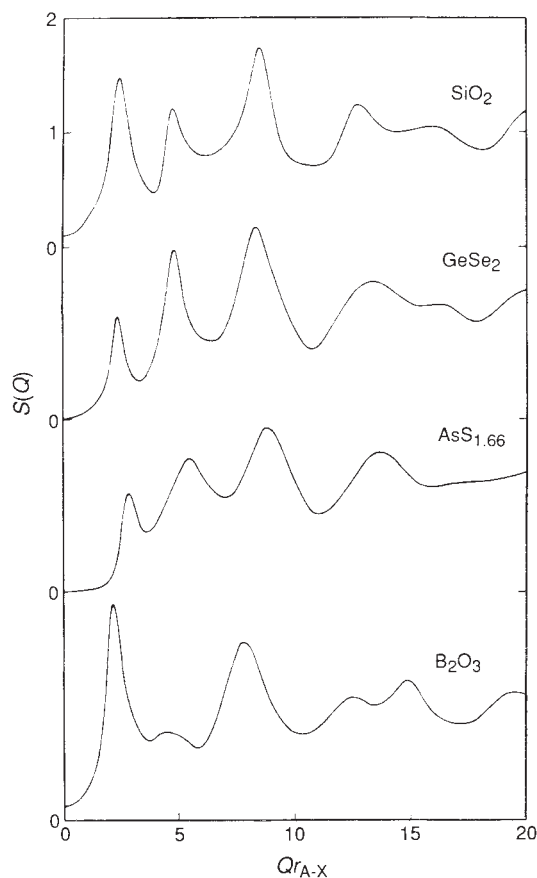


FIG. 6 Structure factor,  $S(Q)$ , for some oxide and chalcogenide glasses plotted<sup>72</sup> against the reduced variable  $Qr_1$ , where  $r_1$  is the nearest neighbour bond length in each case, showing the roughly constant position of the FSDP in this representation.

controlled by the need to minimize the coulombic repulsion between non-bridging oxygens. This condition determines the distribution of  $Q_n$  species, in turn determining the local (SRO) environment of the alkali cations which, in this sense, do not 'modify' the structure at all, their sole effect being to determine the overall number of non-bridging O sites by charge balance.

From a theoretical point of view, computer simulations of structures of amorphous solids should provide considerable insight into the nature and origin of MRO. Progress has recently been made in molecular dynamics simulations by the inclusion of empirical three-body interaction terms in the potential-energy expressions, to try to mimic the effect of non-centrosymmetric (covalent) forces on the structure; this approach has been used, for example, for  $v\text{-SiO}_2$  (refs 61, 62) and  $v\text{-GeSe}_2$  (ref. 10). A more powerful approach is to use potentials that are derived quantum-mechanically in a self-consistent way using first-principles methods, as in the pioneering work of Car and Parrinello (ref. 63); this approach suffers at present, however, from limitations in computing power, which restrict the number of atoms that can be treated. A further limitation of molecular dynamics for simulating glassy structures is that the simulated cooling rates from the liquid state are many orders of magnitude higher than those encountered experimentally. As a result, one must question whether details of subtle structural features, such as those involving MRO, in models resulting from such simulations are truly representative of real glassy materials. But molecular dynamics simulations have the undoubted advantage that, uniquely, they span the transition from liquid to glassy solid and so can be used to ascertain the effect of different types of MRO on the temperature-dependent viscosity behaviour of different systems, and hence ultimately on the glass-forming propensity of liquids.

Finally, a more abstract way of regarding the structure of

noncrystalline materials is as a projection, onto normal three-dimensional euclidean space, of geometric entities ('polytopes') which are regular in a curved, non-euclidean space of higher dimensionality (for a review of this approach, see ref. 64). A formal analogy exists between this approach and that employed to understand the structure of quasi-crystals: in the latter, three-dimensional Penrose tilings—an aperiodic model for the structure—can be generated as a projection from a six-dimensional hypercubic lattice on a three-dimensional hyperplane lying at an incommensurate orientation (see refs 65, 66). In certain cases, symmetries of the polytope will be approximately preserved locally on mapping. For example, rotational symmetries of the polytope in curved space appear as approximate translational symmetries in euclidean space<sup>55</sup>. An intriguing consequence of the existence of regions of such 'polytope-like' order in the structure of real amorphous solids would be that for these domains, an approximately vertical selection rule governing optical absorption should exist<sup>55</sup>, as in crystals:  $(k_f - k_i) \approx 0$  (where  $k_f$  and  $k_i$  are the electron wavevectors corresponding to the final and initial states, respectively). Generally, for noncrystalline materials no such selection rule exists, as the electron wavevector is ill defined in the absence of translational periodicity<sup>1</sup>. Sharp features in the optical (vibrational) spectra of glasses (see, for example, Figs 4 and 5), conventionally regarded as indicating MRO in the form of clusters or rings when vibrational decoupling between modes occurs, could possibly be interpreted instead as a result of polytope-like order. It is interesting to speculate, therefore, to what extent pronounced features of MRO in the structure of real amorphous solids (such as clusters) could be a signature of order in a higher dimensional representation of the disordered structure. □

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# Human immunodeficiency virus genetic variation that can escape cytotoxic T cell recognition

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In a longitudinal study of HIV seropositive patients, there were fluctuations in the specificity of cytotoxic T cells for the virus. This was matched by variability in proviral *gag* DNA epitope sequences in the lymphocytes of these patients. Some of these viral variants are not recognized by autologous T cells. Accumulation of such mutations in T-cell antigenic targets would provide a mechanism for immune escape.

MOST asymptomatic, human immunodeficiency virus (HIV)-infected individuals have cytotoxic T lymphocytes (CTL) that recognize viral antigens presented by HLA class I molecules<sup>1-9</sup>. In these latently infected individuals, HIV-specific CTL activity is very high compared with other virus infections and can often be demonstrated in fresh peripheral blood mononuclear cell preparations without *in vitro* antigen stimulation<sup>1-8</sup>. But once the acquired immunodeficiency syndrome (AIDS) develops, HIV-specific CTL are usually undetectable<sup>1,7,9,10</sup>.

Without experimental evidence, it was thought that persistence of HIV may rely partly on evasion of CTL responses<sup>11,12</sup>. CTL escape mutants have been described only in mice transgenic for a single antilymphocytic choriomeningitis virus (LCMV) T-cell receptor<sup>13</sup>. This model experiment showed that LCMVs that escaped CTL recognition have mutations in the T-cell epitope itself. Here we show that genetic variation in HIV *gag* CTL epitopes in HIV seropositive haemophilic donors can lead to loss of CTL recognition. Accumulation of unseen viral epitopes could be one way by which HIV escapes immune surveillance.

## HIV-seropositive haemophilic donors

Since 1987, we have studied a cohort of HIV-seropositive haemophilic donors. We describe here detailed investigations of CTL recognition of HIV *gag* in six individuals, all of whom were seropositive in early 1985. None have been treated with AZT (Zidovudine). Serial CD4 counts and current disease status of donors 007, 008, 009 and 020 are shown in Fig. 1a. Donors 025 and 049 are siblings with identical HLA types. Donor 025 currently has a CD4 count of  $0.15 \times 10^9$  per litre and is CDC stage III. Donor 049 has a CD4 count of  $0.48 \times 10^9$  per litre and is asymptomatic (CDC stage II). CTL from donors 007, 025 and 049 recognized only a single B27 *gag* protein (Gag) epitope p24-14 and no other epitope in p24 or p17 Gag (ref. 4 and data not shown). CTL from the three other donors 008, 009 and 020 recognize Gag through HLA B8.

## HIV Gag epitope recognition

We have followed HIV Gag-specific CTL in three donors (020,

008, 009) whose response is restricted by HLA B8. We have localized three HLA B8-restricted epitopes in Gag with synthetic peptides, p24-13 (ref. 8), p24-20 and p17-3 (D.F.N., S.R.-J., F.M.G. and A.J.McM., unpublished data). The amino-acid sequences of synthetic peptides used to locate these epitopes are shown in Table 1. Initially (July 1989) both fresh and cultured CTL from donor 020 lysed autologous target cells infected with a recombinant vaccinia virus that expresses HIV-1 Gag p55 (gag-vac). CTL prepared by stimulation of peripheral blood mononuclear cells (PBMCs) with autologous virus expressed in activated T cells<sup>4</sup>, recognized a peptide sequence in the Gag p24 protein (p24-13, residues 255-269) only when peptide-pulsed target cells bore HLA B8 (ref. 8). Later, CTL cultured from blood taken in December 1989 still recognized gag-vac-infected target cells, but this time a second HLA B8-restricted peptide, p24-20 (residues 325-339) sensitized HLA B8-matched target cells (Fig. 1), whereas p24-13 did not. In February 1990 there was another change in the fine specificity of the HIV Gag response. Although CTL from a bulk culture still recognized autologous targets infected with gag-vac (p55), targets treated with p24-13 and p24-20 were poorly recognized. But cells infected with a recombinant vaccinia virus expressing p17 alone were well recognized and the new epitope was mapped using a synthetic peptide, p17-3 (residues 21-35) (Table 1). The p17-rVV and p17 peptides were unavailable in 1989 so it is not known if earlier cultures contained some CTL which could have recognized the Gag p17 epitope. During 1990, CTL from this individual (020) showed a fluctuating response to these three epitopes (Fig. 1b).

In two other donors, 008 and 009, HLA B8-restricted responses to the same three Gag epitopes (p17-3, p24-13 and p24-20) were identified confirming the findings with donor 020. These donors also showed similar fluctuations in the dominant CTL specificities (Fig. 1c, d). Apart from the initial response of donor 020 to p24-13, which was obtained with CTL prepared from fresh PBMCs, all assays required stimulation of the lymphocyte cultures *in vitro* with autologous activated T cells to generate CTL. These responses were therefore driven by autologous virus, rather than added peptide.

In these longitudinal studies, dominant HLA B8-restricted responses apparently shifted from one epitope to another raising the possibility that antigenic variation in the virus might underlie these unexpected changes. There was also evidence of permanent loss of epitope recognition: in two samples from donor 020, (June 1991, July 1991) all detectable CTL responses had disappeared, coincidental with a steady drop in his CD4-positive T cell count (Fig. 1a). By contrast, the CTL responses to the HLA B27-restricted epitope p24-14 in donors 007, 025 and 049 are reminiscent of the sustained CTL responses to influenza virus epitopes that we detected in serial samples from healthy individuals. A.J.McM., F.M.G., S.R.-J., and R. Moots, unpublished observations (Fig. 1d and data not shown).

## Mutations in T-cell epitopes

After defining a single HLA-B27-restricted epitope (p24-14)<sup>4</sup> and three HLA-B8-restricted antigenic epitopes in HIV Gag, we investigated the naturally occurring antigenic variation at these sites *in vivo* by determining the nucleotide sequences of the proviral DNA encoding p17-3 (residues 21-35), p24-13 (255-269), p24-14 (265-279), p24-20 (325-339) (Table 1) and flanking regions. Both briefly cultured and fresh PBL were studied. We deduced amino-acid sequences in HIV Gag and analysed variability across these epitope regions in donors bearing HLA B27 and HLA B8 (Fig. 2). Amino-acid variation in HIV Gag tended to accumulate in or near CTL epitopes and so the site of this variation seemed to be dictated by HLA class I haplotype (Fig. 3).

## CTL do not recognize variant Gag epitopes

We then asked whether this genetic variation in HIV Gag altered CTL recognition. In sequential samples we detected variant amino-acid sequences in all four epitopes (Figs 2 and 3). Pre-

vious studies of the p24-14 epitope, presented by HLA B27, revealed that CTL crossreacted with peptides based on two HIV-1 strains, HIV-2 and one simian immunodeficiency virus (SIV) sequence and the full-length HIV-2 Gag as expressed by recombinant vaccinia<sup>14</sup>. When the p24-14 variant sequence, (Met for Leu at position 270) was tested, this was recognized more efficiently than the original sequence by CTL from donor 007 (Fig. 4) and by CTL from donor 049 (not shown). Thus, there was no evidence of escape mutation in this epitope in these donors.

Comparison of the p17-3 epitope sequences revealed that amino acids 21-24 were invariant in donors 020 and 008 at all time points (Figs 2 and 3). The naturally processed peptide might thus correspond to the variable sequence at amino acids 25-35. Target cells treated with the full-length p17-3 peptide, residues 21-35, the shorter version p17-3', residues 25-35, and a variant sequence p17-3V1 (see Table 1), were all lysed by an effector cell line from donor 020 which was grown, after initial stimulation with autologous activated T cells, on autologous B

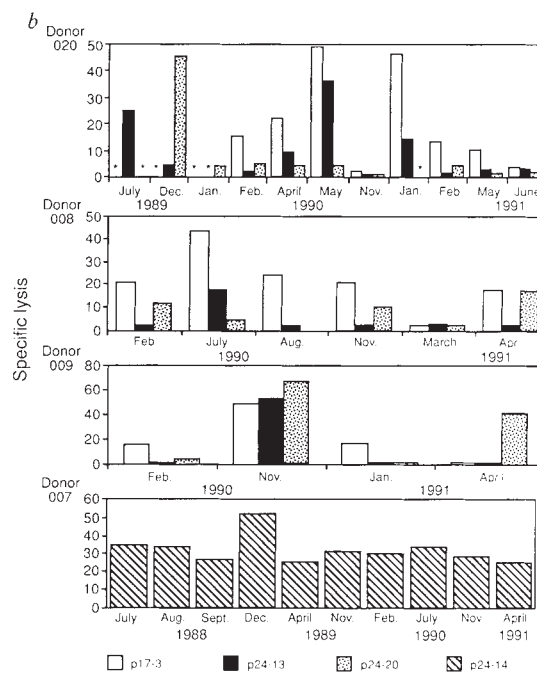
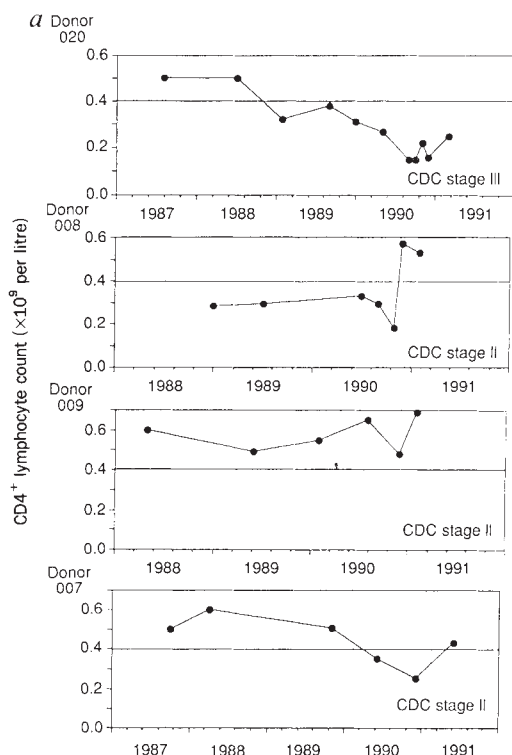


FIG. 1 a, Clinical status of HIV-positive haemophilic donors. Serial CD4 counts and current CDC status of donors 020, 008, 009, 007. CDC stage II is asymptomatic HIV seropositive and CDC stage III is persistent generalized lymphadenopathy. The lower limit of the normal CD4 T cell counts is  $0.4 \times 10^9 \text{ ml}^{-1}$ . The rise in CD4 count of donor 008 in mid-1990 was after he stopped drinking alcohol because of liver damage. 1b, c, d, e, Changing patterns of CTL epitope recognition. Effector CTL recognition of three HLA B8-restricted HIV Gag epitopes in serial samples from donors 020, 008 and 009 compared with CTL recognition of the single HLA B27 Gag epitope 24-14 in donor 007. \*, Not tested.

**METHODS.** *Cytotoxic T lymphocyte preparation.* Heparinised blood (40 ml) was separated on Ficoll Hypaque and the PBMC layer was split into two cultures. The first fraction, which contained one eighth of the PBM was stimulated for 24 h with phytohaemagglutinin (PHA) (Wellcome), and washed twice with a mixture of RPMI 1640 medium (Gibco) and 10% fetal calf serum (R10). This stimulated fraction was then added back to the remaining seven eighths of the culture. These combined cell fractions (the 'bulk' culture) grew in R10 for 7 days and were then treated with lymphocult T at  $10 \text{ units ml}^{-1}$  (Biotest) every 3 days. The assays shown here were tested within 3 weeks of initiating the bulk cultures. *Target Cells* used in the cytotoxic assay were either autologous BCL or HLA class I matched or mismatched BCL. Target

cells were labelled with  $^{51}\text{Cr}$  (300  $\mu\text{Ci}$ , Amersham) for 1 h, washed once with R10 and then incubated with synthetic peptides (concentration range 0.5–50  $\mu\text{M}$ ) for 1 h, washed twice, and resuspended in R10 for 3 h at 37 °C. *Peptide synthesis* was done with solid-phase techniques as previously described<sup>4</sup>. The donor variant peptides were made by Cambridge Research Biochemicals, multi-peptide synthesis. *Cytotoxic assays.* Target cells were washed three times and plated out at either 5, or 10,000 cells per well in 96-well round bottom microtitre plates (Cell-Cult) in 50  $\mu\text{l}$  R10. Effector cells (100  $\mu\text{l}$ ) were added to experiment wells in duplicate. K:T ratios were between 20:1 and 60:1, except for assays of June 1991 in 020, 10:1; November 1990 in 007, 90:1; and April 1990 in 007, 75:1. All assays included control wells for background  $^{51}\text{Cr}$  release (targets with media alone) and maximal  $^{51}\text{Cr}$  release (with 5% Triton X-100) in quadruplicate. After incubating the plates for 4 h at 37 °C, 20  $\mu\text{l}$  supernatant was removed and spotted onto filtermats (Pharmacia), dried in a microwave oven for 4 min and inserted into plastic bags. Beta plate scintillant (10 ml) (Pharmacia) was added to each bag which was then sealed before counting on the LKB Betaplate counter (Pharmacia). Lysis was calculated from the formula: % specific lysis = (experimental counts – media control)  $\times 100$  / (detergent control – media control). Spontaneous release was less than 30% of detergent release.





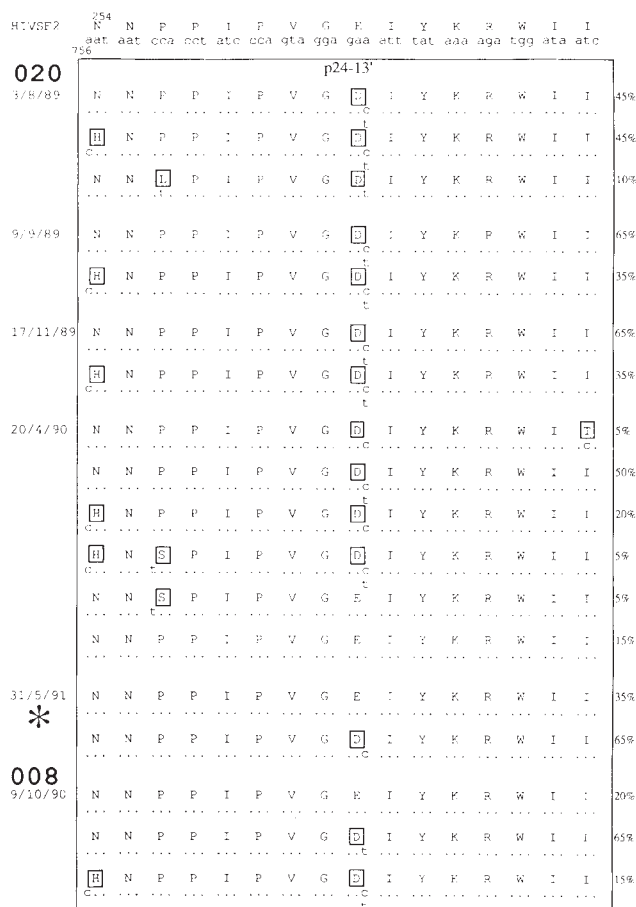
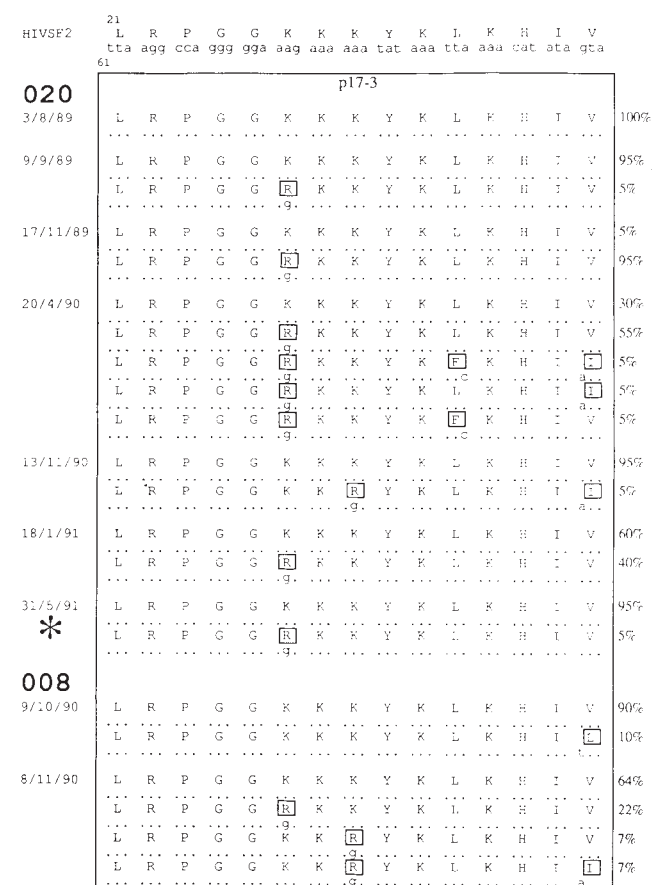
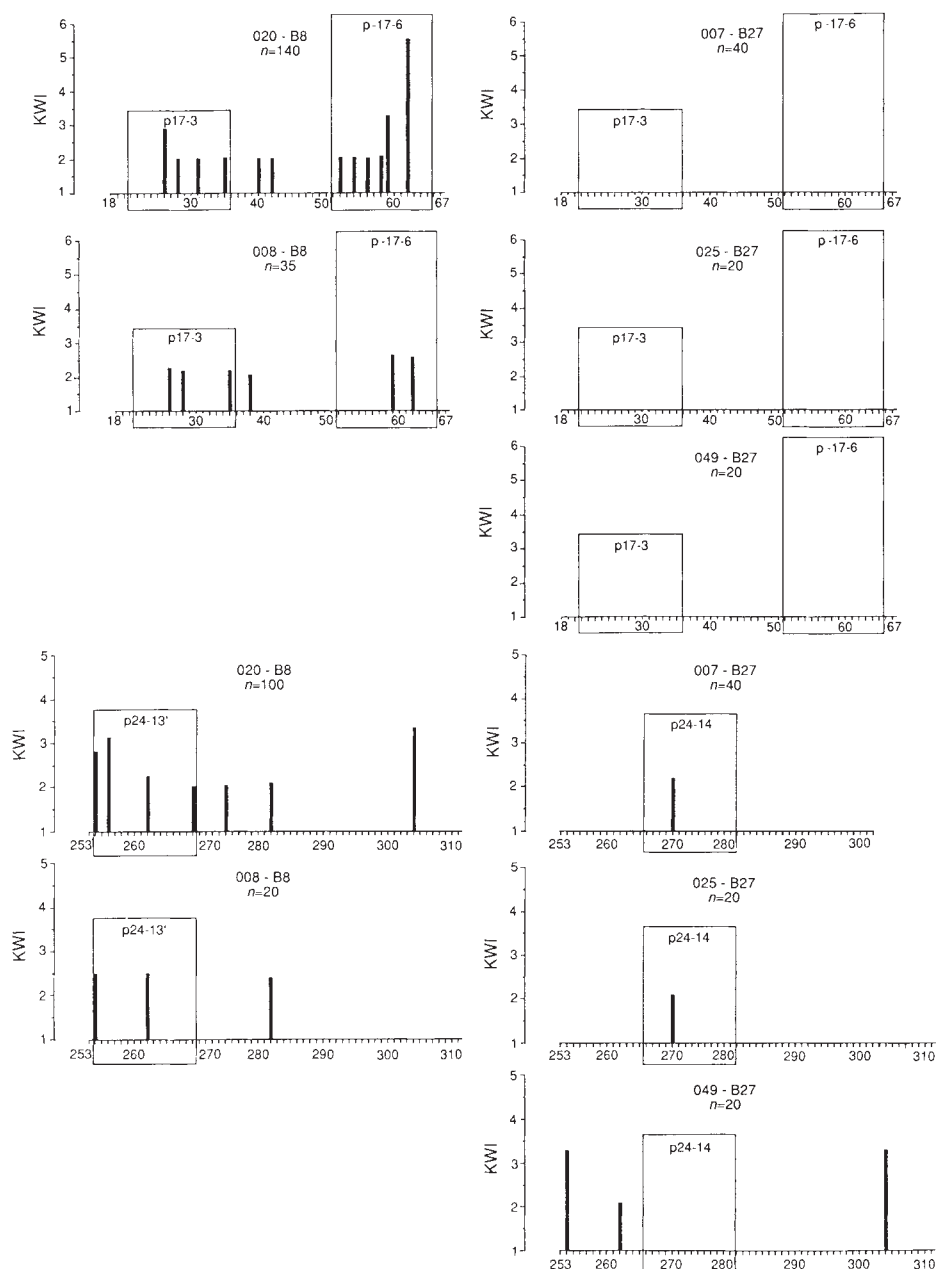


FIG. 2 Sequence variation of HIV Gag CTL epitopes. HIV Gag epitopes p17-3, p24-13, p24-14 and p24-20 in donors 020, 008, 007, 025 and 049 contain variant amino-acid sequences at serial time points (single-letter amino-acid code). At each time all nucleotide substitutions which altered the amino-acid sequence are listed. Data shown are those variants found after deducing the amino-acid sequence for 20 clones at each time point. Variants were only accepted when identified in more than two clones from separate transformations. p24-13' corresponds to epitope p24-13 with the addition of an N-terminal asparagine. Percentage figures in the right hand column refer to the proportion of clones which contained a given variant. \*, Sequences obtained from fresh PBMC DNA amplification.

**METHODS.** DNA was extracted from lymphocytes from the same blood samples used to set up the lymphocyte culture for CTL assays. In preliminary studies, we could not obtain a PCR product visible in an ethidium bromide-stained gel despite 70 cycles of amplification of fresh PBMC DNA, so we used DNA extracted from the PBMC cultured for 1 week in medium alone and used a modified nested PCR technique<sup>29</sup>. We obtained PCR products from seven time points in donor 020 and from three time points in donor 008. To compare the extent of variation we sequenced the region of Gag corresponding to these epitopes from one or two time points in HLA B8 negative donors 007, 025 and 049 whose CTL recognize a dominant Gag epitope corresponding to peptide p24-14, restricted by HLA B27 (ref. 4); their CTL did not recognize peptides p17-3, p24-13 or p24-20. At a given time point, we sequenced 20 clones of M13 for each epitope. Controls containing no added DNA template were used in every PCR reaction at both the primary and secondary step. In June 1991, when his CTL response had disappeared, it first became possible to obtain PCR products from donor 020 after amplification of uncultured PBMC DNA. To validate our findings, we compared sequences amplified from freshly isolated PBMC with those amplified from PBMC after culture for one week *in vitro*. There was no difference in the variants detected by these two methods in either p17-3 or p24-13' at that time (31 May 1991). This indicates that 1 week of culture does not alter the pattern of Gag sequences detected in these epitope regions. Genomic DNA was purified from lymphocytes (Fig. 1) by the Proteinase K method<sup>39</sup>. DNA (1 µg) was added to a buffer containing 0.05 M KCl, 0.001 M Tris pH 8.0, 0.0015 M MgCl<sub>2</sub>, 0.2 mM each of dATP, dCTP, dTTP and dGTP (Pharmacia), 1 µmol of the primary set of Gag-specific oligonucleotide primers (synthesised on an Applied Biosystems 381A DNA synthesiser ▶



FIG. 3 Sites of amino-acid sequence variation in HIV Gag CTL epitope regions. Cumulative HIV Gag-deduced amino-acid sequence variation in regions encoding the HLA B8-restricted epitopes p17-3 and p24-13' from donors 020 and 008 compared with the same coding regions in donors 007, 025 and 049 who recognize the single HLA B27-restricted epitope p24-14 but no other Gag epitope. These data are derived by combining sets of 20 sequences from one to seven time points over 12 to 22 months. Amino-acid positions are numbered according to the Gag reference sequence HIV-SF2. *n*, total number of clones sequenced for each Kabat-Wu index (calculated at each amino-acid residue as the number of different residues/frequency of the commonest residue)<sup>42</sup> (KWI). An index of 1 means that no variation was detected at a particular position. Region p17-6 corresponds to the HIV-SF2 sequence LETSEGCRLQLQ. We deduced the amino-acid sequences of all clones for epitope regions in donors 020 and 008 and compared the variability of sequences encoding the same regions in donors 007, 025 and 049. In donors 020 and 008, amino-acid variation was found in the p17-3 epitope region and in a second site corresponding to amino-acid residues 51 to 65 (peptide 17-6). By comparison, no variation was seen at all in donors 007, 025 and 049 in this region of p17 Gag in a total of 80 HIV Gag DNA sequences. Variation was found in the p24-20 epitope in donor 020 (Fig. 2); in donors 020 and 008 amino-acid variation was also seen in the p24-13 epitope and at Gag position 280 (HIV-1 SF2). In donors 007 and 025, as amino-acid substitution was found in the p24-14 epitope, a region where variability has been described previously<sup>30</sup>. In all three HLA B27 donors there was no variability in the p24-20 epitope region.



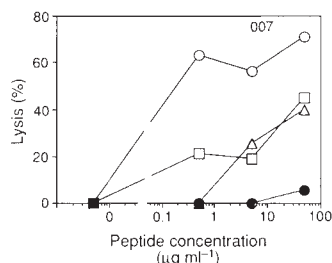
► using cyano phosphoramidite derivatives plus 2.5 units of *Taq* DNA polymerase. Amplifications were always carried out with a positive (HIV-infected CEM cell DNA, a gift from P. Simmonds, University of Edinburgh) and a negative (no DNA control). The amplified fragment was electroeluted into DEAE cellulose in 1% low-melt agarose, digested with *Bgl*II and *Eco*RI and then directionally cloned into M13mp18 cut with the enzymes *Bam*HI and *Eco*RI. Ligated DNA was transformed into *Escherichia coli* JM101 or TG1 by Hanahan's high-efficiency method<sup>40</sup> and clear (recombinant) plaques were sequenced by the dideoxy chain termination method<sup>41</sup>. Cycling conditions for p24-13 amplification were 5 min at 94 °C, 1 min at 50 °C and 30 s at 72 °C followed by 1 min at 94 °C, 1 min at 50 °C and 30 s at 72 °C for 35 cycles. 1% of this reaction product was then used in a second mix which contained a second internal primer pair containing restriction enzyme sites for *Bgl*II and *Eco*RI. p24-13 region primary primers were G3: 5'-ATCAATGAGGAAGCT-3', GEX2: 5'-TCCTTCCACATTCCAAC-3'; secondary (internal) primers *GBgl*II: 5'-GATAGATCTCAAATAGGATGGATG-3', *GEco*RI: 5'-GCTAGAATTCCTGACATGCTGTCA-3'. p17-3 region primary primers 17-3-1: 5'-GGTGGCAGAGCGTCAGTATT-3', 17-3-2: 5'-ACACAATAGAGGGTGCTAC-3'; secondary (internal) primers 17-3*Bgl*II: 5'-GATAGATCTAGAATTAGATCGATG-3', 17-3 *Eco*RI: 5'-GCTAGAATTCGGGATGGTTGTAGCT-3'. PCR amplification conditions were identical to p24-13 amplification except that annealing took place at 55 °C for 1 min during all cycles.

if there were CTL populations that recognized other epitopes presented by the same or other HLA molecules. A small advantage offered by mutation in a single epitope in Gag could well be enough to give that virus a survival advantage. The persistence over time of escape mutant sequences we have documented is strong evidence for that contention. We found that Gag sequences unrecognized by autologous CTL are detectable in seropositive individuals, as shown for the reference p24-13 sequence in donor 020 in April 1990 and May 1991 (Figs 1b and 2) and for the p17-3 variants in donors 020 and 008. New epitopes, if capable of complexing with class I molecules, may stimulate a novel CTL response as for p24-13 variant epitopes (Fig. 6a, b). This may ultimately be harmful if such antigenic diversity drives the immune response to exhaustion as proposed by Nowak *et al.*<sup>28</sup>. But another consequence of antigenic variation would be the generation of an epitope that no longer stimulated a CTL response, as may be the case with the p17-3 variants, possibly because they no longer complex with the presenting HLA class I molecule.

In general, different HLA class I molecules select distinct

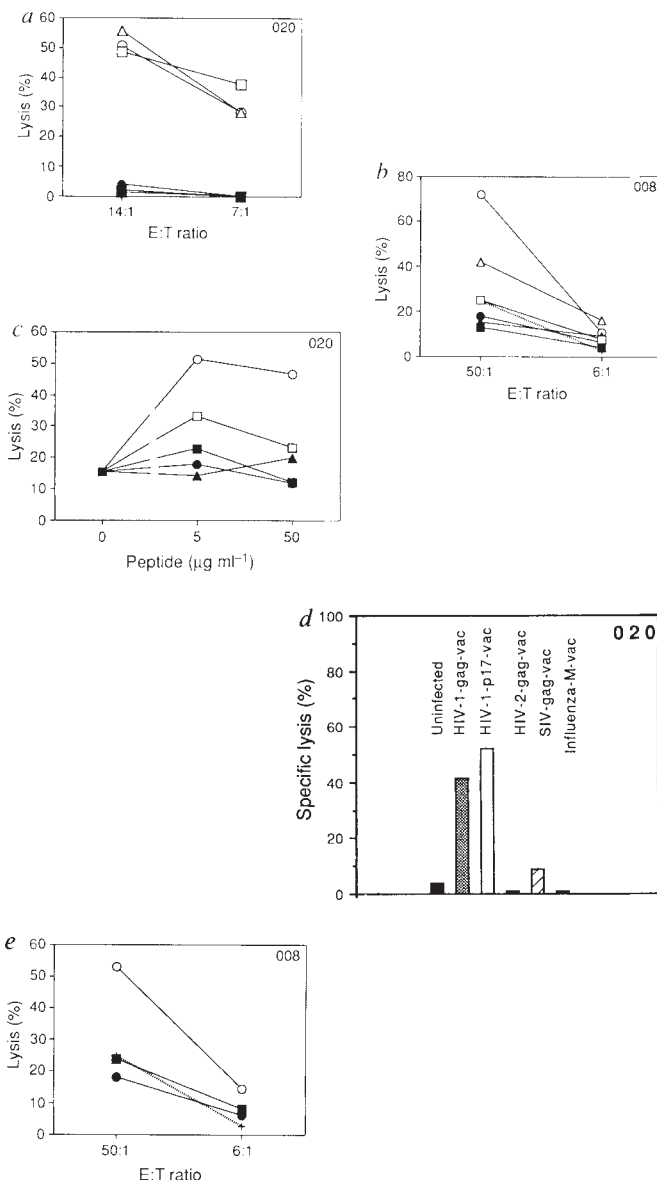
epitopes derived from HIV proteins to stimulate CTL responses<sup>4-8</sup>. Thus HLA type could have an effect on virus escape and possibly clinical progress. Unlike the HLA B8-restricted peptides, less variation was found in the HLA B27-restricted epitope. If an epitope region must be conserved to ensure virus replication or survival, such antigens might elicit sustained CTL responses. If, on the other hand, amino-acid changes in a CTL epitope are tolerated by the virus, then escape mutants might arise. Detailed studies of p24-14 showed that the peptide sequence critically influenced presentation to CTL by HLA B27 (refs 4, 14). But p24-14-specific CTL can crossreact with HIV-2 and SIV sequences and the variant sequence we detected *in vivo* was strongly antigenic; the three residues critical

for recognition [265-267] are invariant in published sequences<sup>29-31</sup>. The relatively high frequency of HIV-amino-acid mutations observed here in the HLA B8 patients, compared with the HLA B27 patients, shows that HIV tolerates sequence changes in all three HLA B8-restricted Gag epitopes. The resulting effect on CTL recognition could help to explain the association of the HLA-A1, B8, DR3 haplotype with poor outcome in HIV infection<sup>32,33</sup>; the effect of HLA B27 on prognosis is not known. HLA B8 is relatively common in caucasians and could exert an effect on variability of prevalent virus strains. Indeed variability in Gag and other relatively conserved HIV proteins other than Env might help to identify peptide epitopes presented to CTL by common HLA types.



**FIG. 5 a**, Donor 020 p17-3-specific CTL fail to recognize targets coated with p17-3 variant peptides. Effectors were a CTL line grown on autologous p17-3-pulsed BCL as described in the legend to Fig. 4. Targets were BCL matched at HLA-B8 only and were preincubated with the peptide for 1 h at a final concentration of  $5 \mu\text{g ml}^{-1}$ . Peptide sequences were as follows: no peptide (●); p17-3, LRPGGKKKKYKLVHIV (○); p17-3', GKKKYKLVHIV (△); p17-3V1, GK[R]YKLVHIV (□); p17-3V2, G[R]KKYKLVHIV (▲); p17-3V3, G[R]KKYK[F]KLVHIV (■). **b**, Donor 008 bulk culture CTL fail to recognize target cells coated with p17-3 variant peptides. Effector cells were from a culture stimulated only with autologous activated T cells as described in the legend to Fig. 1. Target cells were HLA-B8 matched BCL from an HIV uninfected donor. Peptide sequences were as for **a** plus: control peptide influenza nucleoprotein 380-393, dashed line. **c**, CTL from donor 020 fail to recognize published variant p17-3 peptides. CTL were a bulk culture stimulated only with autologous activated T cells as described in the legend to Fig. 1. Targets were HLA-B8 matched BCL from an HIV uninfected donor. The effector:target ratio was 80:1. The peptides used at the concentrations shown were based on published sequences of HIV. Peptide sequences were: p17-3 index HIVSF2, LRPGGKKKKYKLVHIV (○); HIV RF, LRP[R]GKK[R]YKLVHIV (□); HIV CD41, LRPGGKK[R]YKLVHIV (▲); HIV MAN, LRPGGKKKY[R]KLVHIV (■); HIV NDK, LRPGGKKKY[A]KLVHIV (●). **d**, 020 CTL specific for p17-3 fail to recognize HIV-2 Gag and SIV Gag expressed by rVV. CTL from donor 020 tested on autologous target cells incubated with or without rVV-HIV-1 Gag, HIV-2 Gag, SIV Gag, p17 vac or influenza M vac (as a control) for 3 h at a multiplicity of infection of 1, as described<sup>4</sup>. CTL were a line grown on autologous BCL pulsed with p17-3 as described in the legend to Fig. 4. Effector:target ratio was 24:1. Recombinant vaccinia viruses (rVV) were obtained as follows: rVV expressing HIV-gag has previously been described<sup>4</sup>; rVV expressing p17 was made by excising a 317 bp fragment encoding p17 from the plasmid POGS 15 (a gift from S. Adams) with the enzymes *Bam*HI and *Hind*III. The p17 fragment was filled in with Klenow DNA polymerase, ligated into *Sma*I cut pSCII and then inserted into vaccinia virus under the 7.5 K early-late promoter. This recombinant virus contains base pairs 772-1,086 of the HTLV-III B10 Gag sequence including the native Gag start codon and terminates in a stop codon, 33 bp into the vaccinia thymidine kinase gene (position 4,920). The predicted expressed product of this insert is amino-acid residues 1-100 of Gag plus the sequence GNSVSWQTKEK at the carboxy-terminal end. L-D<sup>b</sup> cells infected with this p17-rVV express a product which was immunoprecipitated with the monoclonal antibody ID9 (a gift of B. Ferns, data not shown). The rVV expressing influenza A matrix protein (M-vac), which was used as a control, was a gift of G. Smith and B. Moss (NIH, Bethesda). HIV-2-Gag-Vac and SIV-Gag-Vac were as used previously<sup>31</sup>. **e**, Donor 008 CTL to recognize targets coated with a p24-20 variant peptide. No peptide (●); p24-20', VQNPDPCKTILKAL (○); p24-20V1, VQNPDPCK[R]TILKAL (■); irrelevant peptide (flu NP 380-393), dashed line.

**FIG. 4** CTL from donor 007 recognize p24-14 and a variant sequence (Met→Leu at position 270). Targets were B27-matched lymphoblastoid lines treated with peptides at the concentrations shown as described in the legend to Fig. 1. Effectors were a peptide p24-14-specific CTL: the PBL-derived CTL were grown in the presence of lymphocytic T and autologous Epstein-Barr virus-transformed lymphoblastoid (BCL) feeder cells which had been pulsed with the p24-14 index peptide<sup>4</sup> ( $50 \mu\text{M}$  for 1 h) and irradiated (3,000 rads). K:T ratio was 5:1. Variant p24-14, KRWII[M]GLNK (○); truncated p24-14, KRWIIIGLNK (□); index<sup>4</sup> p24-14, KRWIIIGLNKIVRMYC (△); control p17-6 (●).





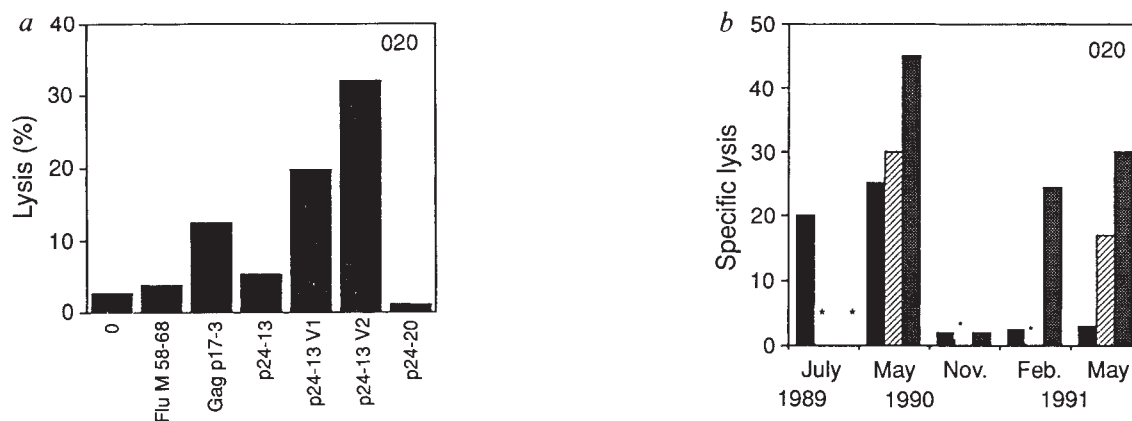


FIG. 6 a, Donor 020 effector cells recognize two p24-13 variant sequences (V1 and V2) but fail to recognize the p24-13' index peptide. Targets are BCL from an HIV-uninfected donor matched at HLA-B8 only and incubated with peptide for 1 h at a final concentration of  $50 \mu\text{g ml}^{-1}$  before use in the assay. Effectors were PBMCs from donor 020 restimulated by the addition of autologous PHA blasts, as described in the legend to Fig. 1. Effector:target ratio was 20:1. Peptide sequences; m-influenza matrix 58-68, GILGFVFTLV; p17-3 15mer, LRPGGKKKYKLKHIV; p24-20,

VQNANPDCKTILKAL; p24-13', NNPPIPVGEIYKRWII; p24-13 V1, NNPPIPVG-[D]IYKRWII; p24-13 V2, [H]NNPPIPVG[D]IYKRWII. b, Changes in 020 effector CTL recognition of p24-13 variant sequences over a 2-year period. CTL were bulk cultures (see legend to Fig. 1), prepared from blood taken on the dates shown, were tested on HLA B8-matched target BCL, pulsed with the peptides shown. Effector:target cell ratios were between 20:1 and 50:1 p24-13', solid bars; p24-13V1, hatched bars; p24-13V2, dotted bars.

Variability in the p17-6 region of Gag amino acids 51-65 is unexplained. Peptides corresponding to these sequences were made and tested with CTL cultured from donor 020 and donor 008 on autologous virus-infected cells but none was recognized (data not shown). Therefore this region does not appear to be a CTL epitope although we cannot completely exclude this possibility. Alternatively, sequence changes outside epitope sequences may affect epitope presentation, possibly by interfering with antigen processing<sup>34,35</sup>. The lack of variation in the p17-6 region of all three HLA B27 donors also supports the idea that accumulation of mutations in the HLA B8 donors was determined by immune pressure.

We propose that variation in HIV Gag CTL epitope regions may be selected by CTL responses. The relationship between HLA B8 and Gag sequence variability in the p17-3 and p24-13 epitopes, which was not found in three B8 negative patients, is striking. Whether this variability is a peculiar feature of B8-restricted responses needs further study, particularly in view of the poor outcome associated with the B8 haplotype. Although some naturally occurring antibodies recognise HIV Gag, including the p17-3 region<sup>36</sup>, gag proteins are not integral membrane proteins and anti-Gag antibodies do not neutralize virus so the conditions necessary for antibody selection of variation do not arise<sup>37</sup>. Although we cannot completely exclude an antibody

selection effect, this variation would not be expected to be directed by HLA class I polymorphism. Although Gag may also be recognized by T helper lymphocytes, these cells, which are not directly involved in virus neutralization or killing, function poorly in HIV-infected individuals<sup>38</sup>.

We have detected variant viral sequences that are not recognized by CTL as anticipated by hypothetical and experimental models of T-cell selection of virus<sup>12,13</sup> and by theoretical predictions of HIV evolution and immune escape<sup>28</sup>. These data indicate a protective role for CTL and have serious implications for vaccine design. Virus variation which evolves *in vivo* may outstrip the capacity of T cells to control the infection. This study supports the idea that protective immunization will require antigen obtained from highly diverse strains of virus. Vaccine strategies which rely on the use of cloned proteins may elicit immunity restricted only to the monomorphic vaccine antigen. Natural infection with HIV would be very likely to overcome this protection as populations of variant sequences unrecognized by neutralizing antibodies<sup>37</sup> and CTL (this study), would have a survival advantage.

*Note added in proof:* Meyerhans *et al.*<sup>43</sup> have recently described sequence variation in the p24-14 epitope in HLA-B27-positive patients but they did not detect accumulation of escape mutants over 8 to 14 months. □

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# Were small galaxies once the dominant cosmological population?

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DEEP sky surveys have turned up unexpectedly large numbers of faint blue galaxies<sup>1,2</sup>—three to five times more than would be estimated by extrapolation from the present galaxy population assuming no evolution. Unless distances to these faint galaxies are known, it is difficult to distinguish between luminosity evolution (in which case, galaxies in the past were systematically brighter) and density evolution (as, for example, if modern galaxies are the results of mergers of earlier ones). We have obtained redshifts and K magnitudes for a small but complete sample of 22 galaxies with B magnitude down to 24. In the luminosity range  $B = 23\text{--}24$ , the B-band galaxy counts are dominated by a population of small blue galaxies at  $z \approx 0.25$ , which may collectively contain as much baryonic matter as the normal galaxies. It is possible either that these earlier galaxies have undergone merging<sup>3</sup> to create the present galaxy population, or that they represent a quite different galactic population which has now faded or disappeared<sup>4</sup>. Either possibility has considerable implications for our understanding of galaxy formation.

The B-band galaxy number counts rise rapidly with magnitude at least to  $B \approx 26$ , at which point the cumulative galaxy density is  $\sim 3 \times 10^5$  per square degree (refs 1, 2). Comparison with a numerical model that assumes no luminosity evolution in the galaxy population and an invariant luminosity function shows that, at  $B \approx 24$ , the actual galaxy counts exceed this model's predictions by a factor of 3 to 5, depending on the assumed local luminosity function or bright count normalization<sup>2,5,6</sup>. In the absence of other information, such as spectroscopy or deep imaging at other wavelengths, the observed counts could be produced in any of three ways: (1) by luminosity evolution in the galaxy population, which increases the distance to which galaxies may be seen in the B band; (2) by the choice of a cosmological geometry that maximizes the available volume; or (3) by increasing the number of galaxies at earlier times, either by introducing additional populations, or by merging, that is by assuming that present-day galaxies were in smaller fragments at this point<sup>2,3,5,7</sup>. The possibility of additional galaxy populations being present has generally been discounted as too radical, although dwarf galaxies have been invoked to explain quasar Ly $\alpha$  absorption lines<sup>8</sup>, and Dekel and Silk<sup>9</sup> have considered the possibility that dwarfs might dominate the baryonic matter and be less correlated than normal galaxies. Models of the B-band counts have therefore concentrated on one or more of the other possibilities. There is considerable freedom of parameters, and it is possible to devise a number of models that fit the data<sup>3,5,7</sup>.

These uncertainties can be resolved to some extent by determining redshifts and types for the galaxies contributing to the B-band counts, which then directly discriminates the origin of the excess. The first attempts to do this<sup>10,11</sup> obtained redshifts for nearly complete samples of galaxies with  $B < 21$  and  $B < 22.5$  and found that the luminosity of the galaxy population did not seem to be evolving substantially: the average redshifts followed predictions of no luminosity evolution rather than increasing more rapidly as would be expected if galaxies were more luminous in the past. This remarkable result has provoked considerable speculation about possible solutions, namely a very-high-volume cosmological geometry dominated by a cosmological constant, rapid galaxy merging at low redshift, starbursting in low-luminosity galaxies or the presence of additional populations of galaxies<sup>4</sup>. But as has been noted<sup>12</sup>, the

TABLE 1 Spectroscopic redshifts of the  $B \leq 24$  galaxy sample

| Object    | B    | K    | z     | $M_K$ |
|-----------|------|------|-------|-------|
| 17 ANON C | 20.0 | 14.7 | 0.211 | -26.1 |
| 17-02     | 21.3 | 17.3 | 0.735 | -26.8 |
| 17 ANON A | 21.4 | 15.7 | 0.288 | -25.9 |
| 22 ANON B | 21.7 | 16.2 | 0.247 | -25.0 |
| 17-01     | 22.0 | 16.8 | 0.644 | -26.9 |
| 17 ANON B | 22.0 | 16.9 | 0.479 | -26.0 |
| 22-K0     | 22.0 | 15.9 | 0.290 | -25.7 |
| 22-01     | 22.1 | 16.5 | 0.301 | -25.2 |
| 13 ANON A | 22.4 | 16.0 | 0.314 | -25.8 |
| 13-K0     | 22.7 | 16.7 | 0.312 | -25.1 |
| 17-03     | 23.1 | 19.7 | 0.202 | -21.0 |
| 22-10     | 23.1 | 21.4 | 0.132 | -18.3 |
| 17-K4     | 23.2 | 19.1 | 0.652 | -24.7 |
| 22 ANON A | 23.2 | 18.6 | 0.536 | -24.6 |
| 17-10     | 23.4 | 20.2 | 0.275 | -21.3 |
| 13 ANON D | 23.5 | 18.3 | 0.449 | -24.5 |
| 17-05     | 23.5 | 19.8 | 0.204 | -20.9 |
| 22-03     | 23.5 | 18.0 | 0.381 | -24.3 |
| 22-16     | 23.5 | 21.0 | —     | —     |
| 13-01     | 23.8 | 18.7 | 0.681 | -25.2 |
| 22-07     | 23.8 | 20.0 | 0.384 | -22.3 |
| 13-04     | 23.9 | 20.1 | 0.274 | -21.4 |

excess over no-evolution predictions is small at these magnitudes, and subtle effects can cause considerable problems with the interpretation.

As we shall describe below, we have extended the spectroscopic sample to  $B = 24$ , where the excess has become very large, and we extend the results of refs 10, 11 by finding that the redshifts continue to fall along the extrapolated no-evolution redshift prediction. But to distinguish between the remaining classes of model, we require yet more information. One important piece of information is the near-infrared luminosity of the galaxy which, being dominated by stars of near-solar mass, measures the old stellar population, as opposed to the blue light which measures transient massive star formation. Measurement of the K (2.2- $\mu$ m) magnitude of the galaxies in the redshift sample shows that the  $B = 24$  excess is primarily caused by an enormous population of small blue galaxies at  $z = 0.2\text{--}0.3$ .

Spectroscopy of a complete sample of 13 galaxies with  $B < 24$  from ref. 2 was obtained using the Canada-France-Hawaii (CFHT) 3.6-m telescope with the University of Hawaii's faint object spectrograph and TI 800  $\times$  800 CCD camera. The procedures used are outlined in detail in ref. 2; briefly, a 2-arcsec-wide, 3-arcmin-long slit was positioned on a nearby bright object and then rotated to fall across several of the galaxies in

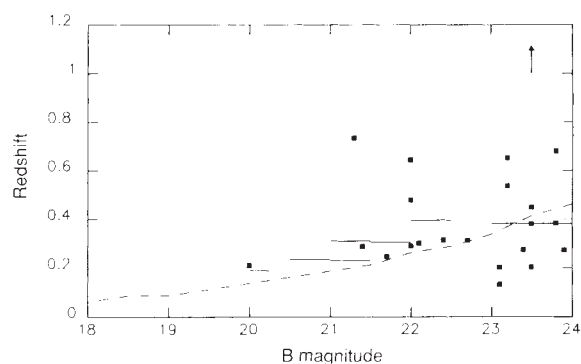


FIG. 1 Redshifts (■) against B magnitude for complete blue spectroscopic sample. Solid lines, median redshifts in various magnitude bins from our sample and from refs 10 and 11; dashed line, predicted mean redshift for a model with no galaxy luminosity evolution.



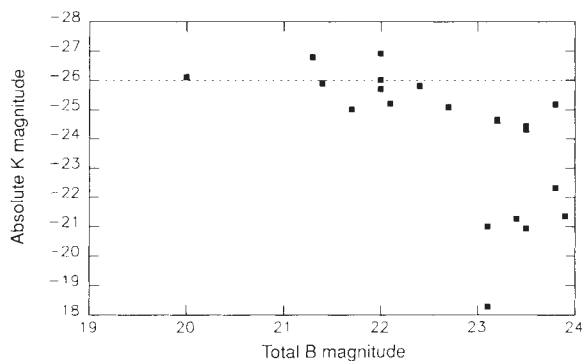


FIG. 2  $M_K \equiv K - 5 \log d_L - 2.5 \log (1+z)$  plotted against B magnitude, calculated with  $H_0 = 50 \text{ km s}^{-1} \text{ Mpc}^{-1}$  and  $q_0 = 0.5$ . The quantity  $M_K$  should approximate the absolute rest-frame K magnitude of the galaxies, as galaxies are nearly flat in  $f_\nu$ , the flux per unit frequency, in the near-infrared. Dashed line, approximate  $M_{K*}$  for elliptical galaxies.

the sample. Several sky-limited exposures were obtained, each typically of 1-hour duration, and between each the field was moved along the slit, which allowed the flat-field pattern and sky background to be determined using median techniques. Total exposure times ranged from 3 to 20 hours, with a typical value of about 4 hours. The final resolution was  $12 \text{ \AA}$  full width at half maximum. Redshifts were measured for all objects with  $B < 24$  that fell on any slit, giving nine objects in addition to those on the original fields. Spectra of some objects have been published previously<sup>2</sup>; the remainder will be published elsewhere, but can also be obtained from the authors. K-band magnitudes were measured for all the objects using the University of Hawaii's NICMOS3  $256 \times 256 \text{ HgCdTe}$  camera on the university's 2.2-m and the CFHT 3.6-m telescopes. The K-band data is described in more detail in ref. 4. The final data set is summarized in Table 1.

In Fig. 1 we show the redshifts of the sample, as well as the median values of the redshifts in different magnitude bins of the present sample and the  $B < 21$  and  $B < 22.5$  samples of refs 10, 11. As was mentioned before, the measured median redshifts agree well with the no-luminosity evolution predictions shown by the dashed line. A more direct interpretation of the data is shown in Fig. 2, where we plot the absolute K magnitude against B magnitude. ( $M_K$  is defined here as  $K - 5 \log d_L - 2.5 \log (1+z)$ , where  $d_L$  is the usual luminosity distance. This assumes that the galaxy spectrum is flat in frequency space, as is very roughly the case in the near infrared. At brighter B magnitudes, most objects have  $M_K \approx -26$  ( $H_0 = 50 \text{ km s}^{-1} \text{ Mpc}^{-1}$ ,  $q_0 = 0.5$ ), as would be expected in a magnitude-limited sample drawn from a Schechter function with  $\alpha = -1.15$  and dominated by ellipticals and early-type galaxies. (The Schechter function is defined mathematically as  $N(L) dL = KL^\alpha e^{-L/L_*} dL$ , where  $N(L) dL$  is the number of galaxies with luminosity between  $L$  and  $L+dL$ . This defines  $L_*$  and  $M_{K*}$  as the corresponding magnitude in the K band.  $L_*$  is the luminosity above which the number density of galaxies drops rapidly.) As we move to fainter magnitudes and higher redshifts, the blue selection chooses late-type spirals and Ims whose  $M_{K*}$  is slightly fainter. More importantly, however, at  $B = 23-24$ , six out of eleven objects have  $M_K$  fainter than  $-22$ , so that at the 95% confidence level, 30–80% of the galaxies are drawn from a low-luminosity population. It is these objects that are primarily responsible for the excess blue galaxy counts. They have  $\langle M_K \rangle_{\text{median}} = -21.3$ ,  $\langle z \rangle_{\text{median}} = 0.24$  and  $\langle (B-K) \rangle_{\text{median}} = 3.4$ , so that they are small blue galaxies with  $\sim 0.01 L_*$  in the near-infrared and lying at very modest redshifts.

The very large number of small galaxies at  $z = 0.25$  is not consistent with expectation if the galaxy population is described

by a luminosity function with an invariant shape equal to that of the local Schechter function. Basically, we need the Schechter function to rise steeply at faint magnitudes. We are therefore left with three options. (1) The B-band luminosity function evolves differentially, with very faint galaxies becoming brighter while bright galaxies remain at constant luminosity<sup>10</sup>. (2) Many present-day  $L_*$  galaxies were fragmented at  $z = 0.25$  and the small galaxies correspond to these fragments<sup>3</sup>. (3) There is a population of dwarfs at  $z = 0.25$  which is not seen today, either because it has faded or, more probably, because it has self-destructed<sup>4</sup>.

The first possibility can be dismissed because the low-mass end of the local luminosity function does not contain enough mass to fuel the production of the observed light. This argument is most easily formulated in terms of the average blue extragalactic light of the low-luminosity galaxy population. If two-thirds of the B galaxy counts fainter than 23 are small galaxies, then the average sky brightness of the population is  $S_\nu \approx 4 \times 10^{-25} \text{ erg cm}^{-2} \text{ s}^{-1} \text{ Hz}^{-1} \text{ deg}^{-2}$ , corresponding to the production of a current metal density of  $(\rho_Z) \approx 1.1 \times 10^{-34} \text{ g cm}^{-3}$  (ref. 14). By contrast, the average baryonic density of material in  $L \leq 0.01 L_*$  galaxies is  $\sim 2\%$  of the total baryonic density in a Schechter function with  $\alpha = -1.15$  where the galaxies have constant mass-to-light ratios, or  $\langle \rho \rangle \approx 10^{-33} \text{ g cm}^{-3}$  (ref. 14). We would therefore require  $\langle Z \rangle$  to have the implausibly high value of 0.1, and we dismiss this hypothesis.

The merging hypothesis is much more attractive although it does face some problems. The small galaxies are too blue to have evolved to an elliptical colour by now, and if they merged to become spirals they would result in objects with much thicker disks than are observed<sup>15</sup>, but these problems could be avoided if they merge into pre-existing much larger galaxies. Measurement of the spatial correlation function<sup>16,17</sup> suggests a much weaker correlation for  $B = 24$  than for the local population. If this result holds up with improved data it would exclude the merger hypothesis, but at present it is only suggestive.

A final possibility is that the new population is completely independent of the normal galaxy population. This hypothesis, if true, would have striking implications. Because the new population contained as much K-band light density as the normal galaxy population, it and its current progeny must contain as much baryonic material as the normal galaxies—more, if dwarfs are gas-rich<sup>13</sup>. The small galaxies rather than the normals were therefore the dominant population at  $z = 0.25$  and, if this population really was less spatially correlated than the normals, our fundamental assumption that the baryonic mass is traced by the normal galaxy light is incorrect. If this is the case, then the implications for our understanding of large-scale structure and our predictions for the microwave background are profound.  $\square$

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# Vibrational spectroscopy of superconducting $K_3C_{60}$ by inelastic neutron scattering

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INTERCALATION of  $C_{60}$  (buckminsterfullerene<sup>1,2</sup>) by alkali metals<sup>3</sup> leads to superconducting compounds of stoichiometry  $A_3C_{60}$  (refs 4–6) with transition temperatures  $T_c$  as high as 33 K (ref. 7). These transition temperatures are considerably higher than those for alkali-metal-intercalated graphite ( $<0.6$  K)<sup>8</sup> and scale with the size of the face-centred-cubic unit cell<sup>9</sup>. Here we present the results of an inelastic neutron scattering study of the vibrational spectrum of the superconducting fulleride  $K_3C_{60}$  ( $T_c = 19.3$  K). We find significant changes in the peak positions and intensities principally of the intramolecular  $H_g$  vibrational modes, both in the high-energy tangential (130–200 meV) and the low-energy radial ( $\sim 50$  meV) regions, compared with the vibrational spectrum of  $C_{60}$  (refs 10, 11). Our results provide strong evidence for the importance of these modes in the pairing mechanism for superconductivity.

The  $K_3C_{60}$  sample was prepared by reaction of  $K_{6.6}C_{60}$  and  $C_{60}$  in a sealed evacuated pyrex tube at 250 °C for 3 days, 350 °C for 24 hours and 250 °C for 8 days<sup>12</sup>. Phase purity was confirmed by X-ray diffraction measurements and Rietveld refinement of high-resolution neutron powder-diffraction data. For the neutron scattering measurements, the 500-mg sample was sealed in a 9-mm-diameter fused silica tube under 0.5 atm helium. Inelastic neutron scattering (INS) measurements were done at 5 K and 30 K with the sample inside a continuous-flow 'orange' cryostat, using the time-focused crystal analyser (TFXA) spectrometer<sup>13</sup> at the ISIS facility, Rutherford Appleton Laboratory; a background run was recorded on an identical empty silica tube at 30 K. TFXA is an inverted-geometry white-beam spectrometer with excellent resolution,  $\Delta\omega/\omega \approx 2\%$ , over the entire energy transfer range, 2.5–500 meV (1 meV = 8.066 cm<sup>-1</sup>). Even

for primarily coherent scatterers like carbon, the instrumental characteristics of TFXA permit the use of the incoherent approximation at energy transfers  $>25$  meV. Coherent scattering effects are important only at low-energy transfer.

The scattering law  $S(Q, \omega)$  of  $K_3C_{60}$  was measured in down-scattering mode between 2.5 and 200 meV. The summed data collected at both 5 and 30 K are shown in Fig. 1.  $S(Q, \omega)$  may be written as

$$S(Q, \omega)_\nu \propto (1/3)(Q^2 \text{Tr} \mathbf{B}_\nu) \exp(-Q^2 \alpha_\nu) \\ \alpha_\nu \approx (1/5)\{\text{Tr} \mathbf{A} + 2(\mathbf{B}_\nu : \mathbf{A} / \text{Tr} \mathbf{B}_\nu)\}$$

where  $\mathbf{A} = \sum_\nu \mathbf{B}_\nu$ ,  $\mathbf{B}_\nu$  is the mean square displacement tensor of the scattering atom in mode  $\nu$  and  $Q$  is the momentum transferred during scattering; other terms and conventions are defined elsewhere<sup>14</sup>. Carbon-60 is a molecular solid with negligible dispersion of the intramolecular phonons, and the observed peaks in  $S(Q, \omega)$  correspond well to the  $Q=0$  modes observed in infrared and Raman spectroscopy. There are no selection rules in INS and hence all the vibrational modes are observed; in addition, considerations of reduced optical penetration depth in  $K_3C_{60}$ , important in Raman and infrared spectroscopy, are not relevant here. The vibrational spectrum of  $K_3C_{60}$ , like that of  $C_{60}$  (ref. 11), may be divided into two regions: (1) 2.5–25 meV, corresponding to intermolecular and  $K^+-C_{60}^-$  modes with a prominent feature at 4.3 meV and a band at 14 meV with full-width at half-maximum of 7 meV; and (2) 25–200 meV, corresponding to the intramolecular modes. The vibrational modes of the molecule may be further subdivided into principally tangential motions, clustering in the high-energy region (110–200 meV) and radial (buckling) motions in the low-energy region (25–110 meV). The symmetry of most of the buckling modes up to 110 meV may be inferred by comparison with  $C_{60}$ ; there are well-resolved bands at 33 ( $H_g^{(1)}$ ), 44 ( $T_{2u}, G_u$ ), 49 ( $H_u$ ), 60 ( $G_g$ ), 68 ( $T_{1u}$ ), 71 ( $T_{1u}$ ), 82 and 87 meV ( $H_g^{(3)}$ ), and a broad band with peaks at 93 and 97 meV ( $H_g^{(4)}$ ). The high-energy region does not show sharp well-defined features and divides roughly into three parts with pronounced cut-offs in intensity: 110–135, 135–170 and 170–200 meV.

Insight into the changes in the intramolecular bonding of  $C_{60}$  on intercalation, and the relevance of the various vibrational modes to the mechanism of superconductivity, can be obtained by comparing the low-temperature INS spectra of  $K_3C_{60}$  and  $C_{60}$ . The striking difference between the two systems is the redistribution of intensity that occurs between the radial and tangential modes. Carbon-60 has intense features up to  $\sim 110$  meV, followed by many weaker and broader bands up to

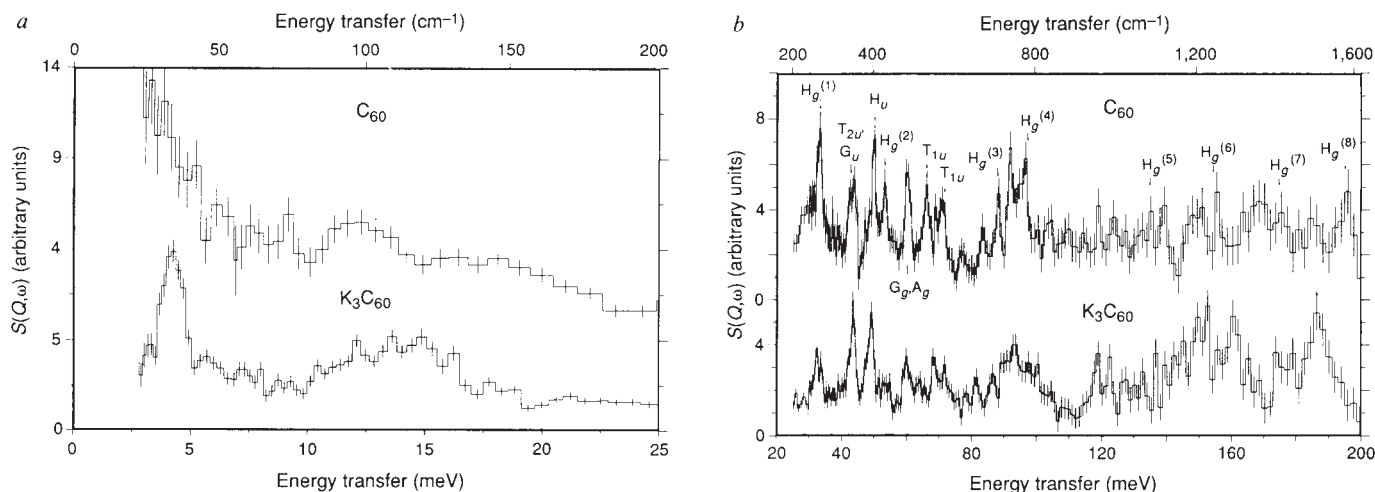


FIG. 1 Inelastic neutron scattering spectra in the energy regions (a) 0–25 meV and (b) 25–200 meV of  $C_{60}$  at 20 K (top) and  $K_3C_{60}$  (bottom); the  $K_3C_{60}$  data were collected at 5 K and 30 K and summed. The instrumental

resolution is  $\Delta\omega/\omega \approx 2\%$ , corresponding to decreased resolution at high energy transfers. Some mode assignments in the icosahedral point group ( $I_h$ ) are shown.



200 meV. In this respect, it resembles graphite which shows the same distribution of intensity between modes involving out-of-plane and in-plane motion<sup>15</sup>. In  $K_3C_{60}$ , on the other hand, the high-energy region is intense. The physical reasons behind these changes in intensity are not clear, but they may reflect increased mixing between radial and tangential modes. One possible mechanism involves the strengthening of interaction force-constants. The effect of this would be to switch intensity between the interacting vibrations, without necessarily changing their energy very much.

Theoretical treatments of superconductivity in  $A_3C_{60}$  have considered purely electronically induced<sup>16</sup> as well as phonon-induced pairing, involving either low-frequency ( $\sim 10$ – $20$  meV)  $K^+-C_{60}^{3-}$  optic modes<sup>17</sup> or the high-energy intramolecular modes<sup>18,19</sup>. In addition, the symmetry considerations for the intramolecular coupling models allow only  $A_g$  and  $H_g$  phonons to couple to the  $t_{1u}$  conduction electrons. Schluter *et al.*<sup>18</sup> find coupling strength distributed over both the low-frequency radial modes and the high-frequency tangential modes, whereas Varma *et al.*<sup>19</sup> find that the purely tangential  $H_g^{(7)}$  and  $H_g^{(8)}$  modes at 177 and 196 meV dominate the pairing mechanism. Strong electron-phonon coupling will produce substantial broadening and softening ( $\sim 10$ – $20\%$ ) of the affected modes. Raman spectroscopy is sensitive to the  $A_g$  and  $H_g$  modes with both  $A_g$  and all eight  $H_g$  modes evident for  $C_{60}$ . Room-temperature Raman spectra<sup>20</sup> of  $A_3C_{60}$  thin films show only two  $A_g$  modes and the lowest-frequency  $H_g^{(1)}$  mode, the absence of the other modes being attributed either to decreased optical penetration depth or lifetime broadening of the spectral function due to electron-phonon coupling.

The INS data confirm the electron-phonon coupling to at least some of the  $H_g$  modes. Indeed the most remarkable difference between the  $K_3C_{60}$  and  $C_{60}$  spectra is the disappearance of the  $H_g^{(2)}$  and  $H_g^{(8)}$  modes at 54 and 196 meV, consistent with strong electron-phonon coupling. This is an important difference from alkali-intercalated graphite, where the corresponding buckling  $H_g^{(2)}$  mode does not couple to the  $\pi$ -electrons because of symmetry considerations. Radial  $H_g^{(1)}$ ,  $H_g^{(3)}$  and  $H_g^{(4)}$  modes are present in  $K_3C_{60}$  at 33, 87 and 97 meV and show only slightly reduced intensities and increased broadening, consistent with minor contributions to the electron-phonon coupling strength. A definitive statement about the effect of intercalation on the  $H_g^{(5-7)}$  modes at 136, 155 and 177 meV in  $C_{60}$  is difficult because they are weak, but there are significant changes in the spectra of  $K_3C_{60}$  in these regions. Although definitive peak assignments must await detailed modelling, it is tempting to assign the broad feature in  $K_3C_{60}$  at 186 meV to  $H_g^{(8)}$ , corresponding to a softening of  $\sim 6\%$ . The differences between  $C_{60}$  and  $K_3C_{60}$  in the 10–20 meV region may be due to  $K^+-C_{60}^{3-}$  optic modes.

We have also recorded the INS spectra of  $K_3C_{60}$  above (30 K) and below (5 K) the superconducting transition temperature. There is only modest evidence for changes in the intensity of the intramolecular modes in the 135–170 and 170–200 meV regions, as the sample goes through  $T_c$ . A decrease in intensity is, however, observed between 30 and 5 K at 4.2 meV; there is also some indication that this is accompanied by an increased broadening. Note that the scattering in this energy range is completely coherent, and we are cutting through a phonon, probably acoustic, branch. These effects may be caused by an as yet undetected structural anomaly at or above  $T_c$ , or arise because the phonon energy is close to the superconducting energy gap ( $2\Delta \approx 40$  K (ref. 21)). We note that the presence of soft acoustic phonons is well documented in the case of A15-structure superconducting materials<sup>22</sup>.

Our data provide strong evidence for the presence of electron-phonon coupling to  $H_g$  modes of  $K_3C_{60}$ , as predicted by intramolecular phonon theories<sup>18,19</sup>. Higher-resolution data and detailed mode assignments are needed for a more complete picture, particularly the location of the softened  $H_g$  modes in

$K_3C_{60}$ , identification of optic modes and the nature of the near-gap scattering law below  $T_c$ . □

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## Layered magnetic structure of a metal cluster ion

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THE ability of molecular materials to perform many of the optical, electronic and magnetic functions traditionally associated with extended two- and three-dimensional inorganic solids<sup>1,2</sup> has given rise to intensive research on molecular electronics<sup>3,4</sup>. In the course of investigating the properties of a class of anionic metal clusters based on the vanadium oxide systems<sup>5–8</sup>, which bear analogy with those of bulk solid materials<sup>6</sup>, we have encountered unusual magnetic behaviour in a finite molecular system. A cluster containing 15 paramagnetic vanadium atoms consists of three distinct layers in each of which the magnetization shows a distinct temperature dependence. Analogous behaviour in bulk systems can be found in magnetic multilayers<sup>9</sup> and also in copper oxide superconductors, where copper layers with strong antiferromagnetic coupling are separated by layers of rare-earth ions in which the coupling is very weak<sup>10</sup>. The behaviour of this cluster suggests the possibility of applications for molecular-scale switching.

$K_6[V_{15}As_6O_{42}(H_2O)] \cdot 8 H_2O$  was prepared as described previously<sup>11</sup>. It has a crystallographically imposed trigonal symmetry<sup>11</sup> (space group  $R\bar{3}c$ , No. 167), as shown in Fig. 1a. The overall structure is quasispherical, with three sets of (non-equivalent) vanadium atoms,  $V_1$ ,  $V_2$  and  $V_3$ .  $V_1$  and  $V_2$  define two nonplanar hexagons separated by a triangle of  $V_3$  centres (Fig. 1b). This 'layer structure' has paramount importance in determining the magnetic properties to be described below.

Figure 2 shows the temperature dependence of the effective magnetic moment per formula unit,  $\mu_{eff} \propto (\chi T)^{1/2}$ , where  $T$  is temperature and  $\chi$  is magnetic susceptibility of  $K_6[V_{15}As_6O_{42}(H_2O)] \cdot 8 H_2O$  in the range of 5–300 K, measured with a Faraday apparatus<sup>12</sup>. The room-temperature value,  $4.05 \mu_B$ , is much lower than expected for 15 uncoupled

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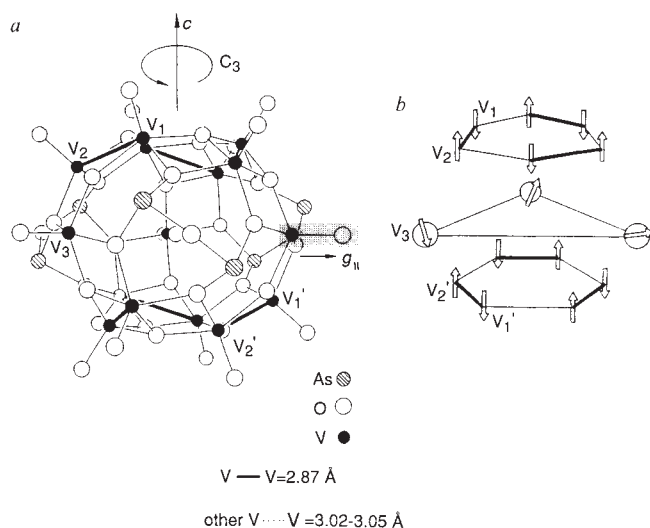


FIG. 1 *a*, Structure of the  $[V_{15}As_6O_{42}(H_2O)]^{6-}$  cluster<sup>11</sup> with the crystallographic  $c$  axis (hexagonal setting with  $a$  ( $=b$ ) perpendicular to  $c$ ) and explanation of the notation  $g_{\parallel}$  as defined within the plane of the three uncoupled V(=O) centres indicated. *b*, Schematic sketch of the proposed spin arrangement at lower temperatures (see text). Arrows in circles correspond to the uncorrelated spins ( $V_3$  sites). The magnetic layers are defined by the two nonequivalent planes (perpendicular to the  $c$  axis) through the centres of the  $V_1$ - $V_2$  vectors and the  $V_3$  centres, respectively. The distance between these planes is 2.56 Å. (The distances of  $V_1$  and  $V_2$  to the averaged hexagon plane are 0.41 Å.)

oxovanadium(IV) ions,  $\mu_{\text{eff}} = [n(n+2)]^{1/2} N^{1/2} \mu_B = 6.7 \mu_B$ , where  $N$  is the number of V atoms and  $n=1$ , indicating the presence of antiferromagnetic interactions. The effective magnetic moment rapidly decreases with decreasing temperature, reaching a plateau below 100 K which corresponds well to the value expected for three uncoupled electrons ( $3.1 \mu_B$ ). Below 20 K, the moment decreases slightly.

We interpret these data as indicating a strong coupling within the  $V_1$ - $V_2$  pairs in the hexagons which leaves the three  $V_3$  centres largely uncoupled. The final proof of this assumption comes from the single-crystal electron paramagnetic resonance spectra<sup>13</sup> recorded at 25 K, which are shown in Fig. 3. They give electronic  $g$ -factors of  $g_a = g_b = 1.95$ ,  $g_c = 1.98$ , where  $a$ ,  $b$  and

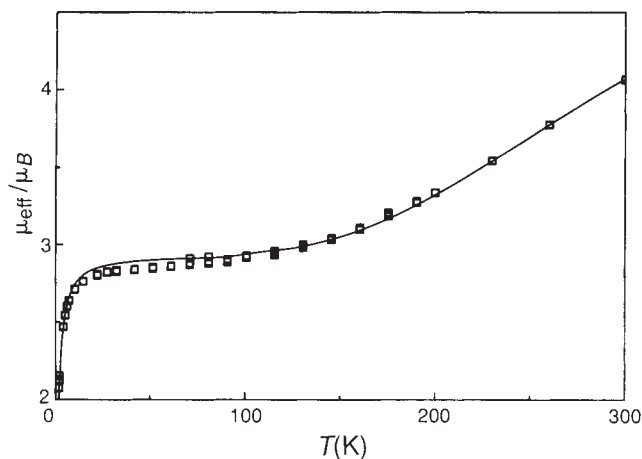


FIG. 2 Magnetic moment of  $K_6[V_{15}As_6O_{42}(H_2O)] \cdot 8 H_2O$  in the 5–300 K temperature range. The curve has been calculated as described in the text.

$c$  are the crystallographic axes (Fig. 1). These values do not correspond to those usually observed for oxovanadium(IV) ions, which have axial tensors<sup>14</sup> with  $g_{\parallel} = 1.93$  and  $g_{\perp} = 1.98$  (but  $g_{\perp} < g_{\parallel}$  also occurs<sup>15</sup> in a comparable species  $[Mo_{10}V_2PO_{40}]^{6-}$ ). The subscripts  $\parallel$  and  $\perp$  indicate directions parallel and perpendicular to the (short) terminal V=O bond respectively (Fig. 1).

In the structure of  $K_6[V_{15}As_6O_{42}(H_2O)] \cdot 8 H_2O$ , the three  $V_3$  centres have  $g_{\parallel}$  orthogonal to the trigonal axis, and the average of the three sites in this plane yields  $g_a = g_b = \sqrt{(g_{\parallel}^2 + g_{\perp}^2)}/2 = 1.95$ . On the other hand, the  $V_3$  centres have  $g_{\perp}$  parallel to  $c$ , and therefore  $g_c = g_{\perp} = 1.98$ , in excellent agreement with experiment. If the  $g$  tensors of the  $V_1$  and  $V_2$  ions also contributed, the overall  $g$  tensor of the cluster would be isotropic, contrary to the experimental findings.

These results indicate that the cluster is an effective and novel type of multilayer magnetic structure, with three arrays of different magnetization which can be easily recognized below 100 K. In Fig. 1*b* we sketch the proposed spin arrangement at low temperature: within the  $V_1$ - $V_2$  pairs, the spins are completely paired, whereas the spins in the  $V_3$  planes are uncorrelated, which corresponds to spin frustration.

If we assume that the strongest coupling constant,  $J$ , is related to the  $V_1$  and  $V_2$  centres, which are separated by only 2.87 Å (each  $V_1$  ion is principally coupled to two  $V_2$ , but the two  $V_1$ - $V_2$  distances are quite different from each other, 2.87 Å and 3.05 Å respectively, and this causes the spin pairing in the three  $V^{IV}$ - $V^{IV}$  pairs of the two hexagons), we can distinguish two temperature domains for the magnetic susceptibility. The high-temperature domain ( $T > 100$  K) is described taking only the strong coupling  $J$  into account, and suggests  $J \approx 10^3$  K. The spins on  $V_3$  are uncorrelated, not because they are not coupled to  $V_1$  and  $V_2$ , but because of the strong  $V_1$ - $V_2$  coupling.

In the low-temperature range ( $T < 100$  K), we take all the other couplings ( $V_2$ - $V_2'$ ,  $V_1$ - $V_3$ ,  $V_2$ - $V_3$  and  $V_1$ - $V_2'$ ) into account in a perturbation treatment. To obtain an effective coupling between the  $V_3$ , the perturbative approach has to be extended to third order. This is because the interaction between the  $V_3$  and  $V_2'$  involves three weak couplings, namely  $V_3$ - $V_2$ ,  $V_2$ - $V_2'$  and  $V_2$ - $V_3$ . Thus the two doublets and the quartets associated with the three  $V_3$  spins are slightly split, producing the decrease of magnetic susceptibility below 20 K. The splitting is given by  $E = 3/4 [J(V_1-V_3) - J(V_2-V_3)]^2 [J(V_1-V_2') - J(V_2-V_2')]/J^2$ , where  $J(V_1-V_m)$  is the coupling between the indicated vanadium atoms. The best fit to the magnetic data, shown in Fig. 2, was obtained for  $J = 800$  K, and  $J(V_1-V_3) - J(V_2-V_3) =$

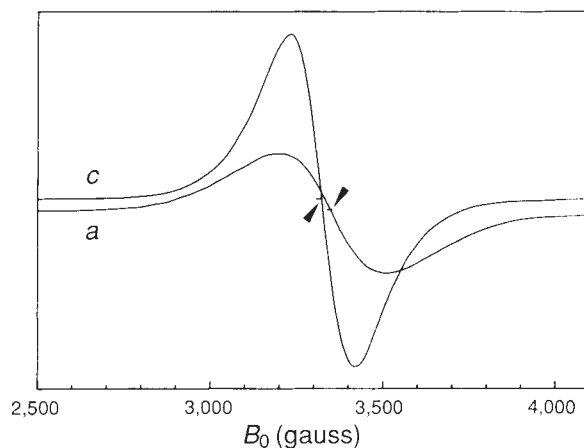


FIG. 3 Single-crystal electron paramagnetic resonance spectra of  $K_6[V_{15}As_6O_{42}(H_2O)] \cdot 8 H_2O$  recorded at 25 K, giving  $g_a = g_b = 1.95$  and  $g_c = 1.98$  (see text). The vertical axis denotes absorption intensity. The arrowheads indicate the  $g_a$  and  $g_c$  values.



$J(V_1-V_2) - J(V_2-V'_2) = 120 \text{ K}$  (the identity of the left- and right-hand sides of the equation is also justified according to the geometry of the cluster).

The cluster  $[V_{15}As_6O_{42}(H_2O)]^{6-}$  can be regarded as a first step towards vanadium-oxygen models for molecular electronics, the aim of which is to use molecules as very small switching elements to achieve information storage or signal processing<sup>3,4</sup>. The requirement that a molecular system should fulfil is that it is able to change its electronic ground state as function of a perturbation<sup>4</sup>. This is especially interesting because our findings are related to the discontinuous phase transition in  $VO_2$ . In monoclinic  $VO_2$  (low-temperature modification with distorted rutile structure), the V-V distances along the *c*-axis are not, as in  $TiO_2$ , of equal length but alternately short and long with spin pairing within the short  $V^{IV}-V^{IV}$  pairs, as in the hexagons of  $[V_{15}As_6O_{42}(H_2O)]^{6-}$  at low temperatures. On heating above  $67.5^\circ\text{C}$ , the  $V^{IV}-V^{IV}$  pairs 'break apart' (transition into the tetragonal rutile structure) to form antiferromagnetic coupled centres again as in the cluster at high temperature. (In  $VO_2$  there is also a jump in the conductivity at the phase transition from a semiconductor to a metallic conductor<sup>16</sup>.) The range of variation shown by vanadium-oxygen clusters, from weak exchange interactions through very strong exchange interactions to relatively strong covalent bonds seems unique in clusters with exclusively 'classical' ligands such as  $O^{2-}$  (ref. 6).

(The magnetic properties seem to correlate with the bond data and with the presence of  $V^{IV}-V^{IV}$  pairs as 'spin traps')<sup>6</sup>. Therefore different types of bistability and/or multistability (with respect to the electronic structure) may be expected and designed in vanadium-oxygen clusters, a promising step towards the development of a molecular switch. Our system is particularly easy to understand, as we are dealing only with electronically simple  $d^1$  centres. □

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## Tracing the conversion of aurichalcite to a copper catalyst by combined X-ray absorption and diffraction

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EVER since X-ray sources first became available, the merit of deploying diffraction and absorption spectroscopic studies simultaneously has been acknowledged<sup>1</sup>. Information on oxidation states and local ( $\sim 6\text{-Å}$  radius) atomic environments is now obtained routinely from X-ray absorption measurements using synchrotron sources<sup>2-4</sup>. Synchrotron radiation is also used commonly for high-resolution powder diffraction crystallography. We report here an instrumental arrangement that has allowed us to extract quantitative short- and long-range structural information on samples undergoing chemical change by measuring X-ray absorption spectra and X-ray diffraction patterns *in situ* and within a few seconds of one another, using a synchrotron X-ray source. To illustrate the combination of these techniques, we have followed the structural and chemical changes that occur within the layered mineral aurichalcite ( $Cu_{2-x}Zn_x(OH)_6(CO_3)_2$ ) when heated in dry air to  $\sim 450^\circ\text{C}$ . Despite marked changes in crystallinity, the local environment and electronic state of the  $Cu^{2+}$  ions remain unchanged, even when at  $\sim 450^\circ\text{C}$  the material is converted to a mixture of  $CuO$  and  $ZnO$ . Heating this mixture in  $H_2/N_2$  produces an active catalyst for the water-gas shift reaction ( $CO_2 + H_2 \rightarrow CO + H_2O$ ), which our studies show to consist of small particles of copper metal (with some zinc incorporated) supported on  $ZnO$ .

By using a fixed arrangement (see Fig. 1) in which an energy-

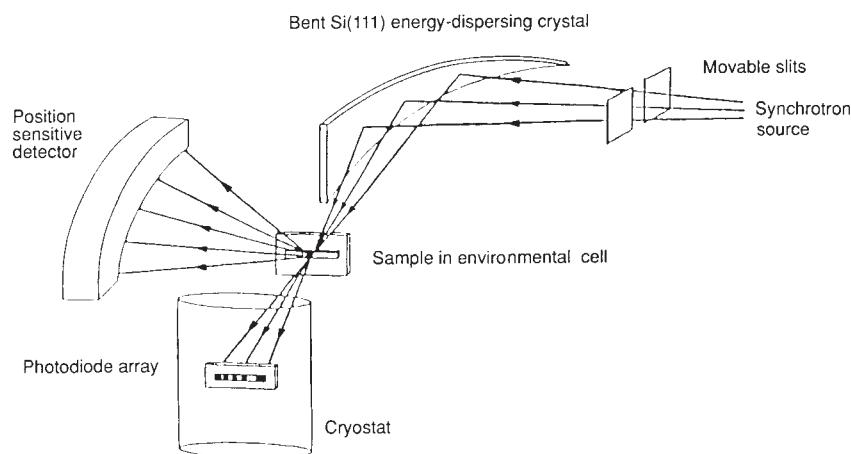
dispersed geometry developed for dynamic X-ray absorption studies<sup>5-7</sup> has been augmented with a curved, position-sensitive detector, we have recorded low-resolution X-ray diffraction patterns and absorption spectra in parallel on the same sample under identical environmental conditions. The dispersive absorption measurements were carried out on station 7.4 of the Daresbury SERC synchrotron radiation source using a bent triangular silicon crystal which disperses the incoming white X-ray beam and focuses it horizontally.

The arrangement of a dispersive monochromator and photodiode array (Fig. 1) is well suited for quantitative, dynamic absorption studies, as we have shown<sup>8</sup> for the thermal dehydration of  $Ni^{2+}$ -exchanged samples of zeolite Y and *in situ* reduction of nickel oxide supported on alumina (unpublished). (Large changes both in fine-structure oscillations and in chemical shifts of the absorption thresholds were readily discerned.) Details of the data acquisition and features of the design and operation of the photodiode array used here (a cooled RL1024S Reticon array) are given elsewhere<sup>9</sup>.

X-ray diffraction patterns from powdered samples were conveniently measured alongside energy-dispersive absorption spectra using the curved position-sensitive detector placed vertically above the specimen stage at the focus of the monochromator. Although, in principle, diffraction and absorption patterns may be collected simultaneously, in practice the best results are obtained by recording diffraction and absorption sequentially in the same experiment with a very short time delay. This arises because the large bandpass,  $\Delta E$ , required for absorption spectra, broadens the width of the diffraction peaks and also causes interference from fluorescence, thereby adding to the background noise of the diffraction patterns. These effects can be eliminated by making the measurements in sequence with different  $\Delta E$ s. A movable slit is therefore included which opens when the photodiode array is operating and closes when the curved position-sensitive detector is in operation. We chose a band pass of 250 eV for absorption, with a reduction to 30 eV for diffraction. The mean Bragg angle of the monochromator  $\Theta_{av}$ , for diffraction is chosen so that the photon energies are insufficient to excite X-ray fluorescence for the element under investigation. The reduced bandpass for diffraction results in a smaller focusing angle,  $\Delta\Theta$ , of  $0.05^\circ$ . Although this exceeds the monochromator Darwin width and the source size contribution

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FIG. 1 Schematic diagram of the experimental set-up used for combined X-ray absorption spectroscopy and X-ray diffraction *in situ* measurements. The Si energy-dispersing crystal subtends an angle  $\Delta\theta$  at the sample given by  $\Delta\theta = \tan \Theta_{av}(\Delta E/E)$ , where  $\Theta_{av}$  is the mean Bragg angle of the monochromator. For a dispersed bandpass of 250 eV at 8.3 keV, for example, the focusing angle,  $\Delta\theta$ , of a Si{111} monochromator ( $\Theta_{av} = 13.8^\circ$ ) is  $0.43^\circ$ .  $\Theta_{av}$  is chosen so as to straddle the absorption threshold,  $E$ , of the element under investigation, and the transmission spectrum is recorded on a photodiode array which is typically placed  $\sim 1$  m beyond the sample stage<sup>8,13</sup>. The spectral resolution  $\delta E/E$  is governed by the angular dispersion  $\delta\theta$  for a given  $\theta$ , where  $\delta\theta = \tan \theta(\delta E/E)$ . Bearing in mind that  $\delta\theta$  is the convolution of the Darwin width of the monochromator, estimated to be  $3.4 \times 10^{-5}$  for  $13.8^\circ$ , with the angle subtended by the source ( $1.8 \times 10^{-5}$  for station 7.4) and the angle subtended by a 25- $\mu\text{m}$  pixel (typically  $3 \times 10^{-5}$ ),  $\delta\theta \approx 5 \times 10^{-5}$ . We see, therefore, that for a Si{111} monochromator the corresponding spectral bandpass at 8.3 keV is  $\sim 2$  eV at station 7.4. The photon intensity at the focused spot in this geometry is  $\sim 10^{10} \text{ s}^{-1}$ , the size of the spot being close to 0.5 mm horizontally. With no vertical focusing available on this station (see text) the height of the spot is set by an entrance slit to  $\sim 1$  mm to match the angular resolution of the curved position-sensitive detector



and also the aperture of the environmental cell. The specimens studied here transmitted  $\sim 10\%$  of incident radiation and were 50–100  $\mu\text{m}$  thick; the beam itself intersects  $\sim 100 \mu\text{g}$  of specimen. The X-ray range available on station 7.4 is  $\sim 7$ –12 keV, these bounds corresponding to the limits set by the air-scatter for diffraction measurements and the spectral range of the synchrotron radiation cut-off from the dipole magnet source, respectively<sup>14,15</sup>.

to the angular dispersion, it is well matched to the angle subtended by the specimen at the curved detector and by the intrinsic angular resolution of the detector. The INEL detector used offers an angular range of  $120^\circ$  and an angular resolution of  $0.04^\circ$  (or  $7 \times 10^{-4}$  rad). Powder patterns of the quality shown here can be obtained in 2–3 min on station 7.4. Further details of the instrumentation for combined absorption–diffraction are given elsewhere<sup>9,10</sup>.

We illustrate the power of combined absorption–diffraction studies by tracing the conversion of the catalyst precursor aurichalcite, a mineral phase whose structure is unknown but thought to be akin to that of the layered mineral hydrozincite ( $\text{Zn}_5(\text{OH})_6(\text{CO}_3)_2$ ), to an active Cu-on-ZnO catalyst which facilitates methanol formation (from  $\text{CO} + \text{H}_2$  mixtures) and reverse water–gas shift ( $\text{CO}$  and  $\text{H}_2\text{O}$  from  $\text{CO}_2$  and  $\text{H}_2$ ) (ref. 11, and D.W., in preparation). Separate experiments using a range of characterizing techniques including thermal analysis and ultraviolet, and infrared spectroscopies, have established that aurichalcite, when precipitated by addition of 1.5 M solutions of sodium carbonate at  $\sim 60^\circ\text{C}$  (pH = 7), forms a stable phase of Cu/Zn ratios ranging from 0.25 to 1.0. Higher and lower ratios of Cu/Zn favour formation of malachite and hydrozincite, respectively. In synthetic samples of aurichalcite the  $\text{Cu}^{2+}$  and  $\text{Zn}^{2+}$  seem to be distributed over the two octahedral and one tetrahedral sites, with the  $\text{Cu}^{2+}$  preferentially occupying octahedral sites. Phase characterization of the precursor solid entailed a combination of conventional powder diffraction (which also yielded unit-cell parameters), thermal analysis, electron microscopy and electron diffraction. Surface areas of the as-prepared and calcined samples were determined by the nitrogen BET procedure, and that of the copper metal in the reduced (active) catalyst from the amount of  $\text{N}_2\text{O}$  stoichiometrically converted to  $\text{N}_2$  (ref. 11).

The reverse water–gas shift reaction was studied by mass spectroscopy using a flow system with 1 bar pressure of  $\text{CO}_2$ ,  $\text{H}_2$  and He (1:1:8 mixture) passing through the environmental cell, which permitted simultaneous study by synchrotron radiation. This cell, which consists of a custom-built Kanthal heating element embedded in a pyrophyllite block with a recess to accommodate the compressed specimen, could be heated linearly from room temperature to  $800^\circ\text{C}$  under vacuum or in a gas flow.

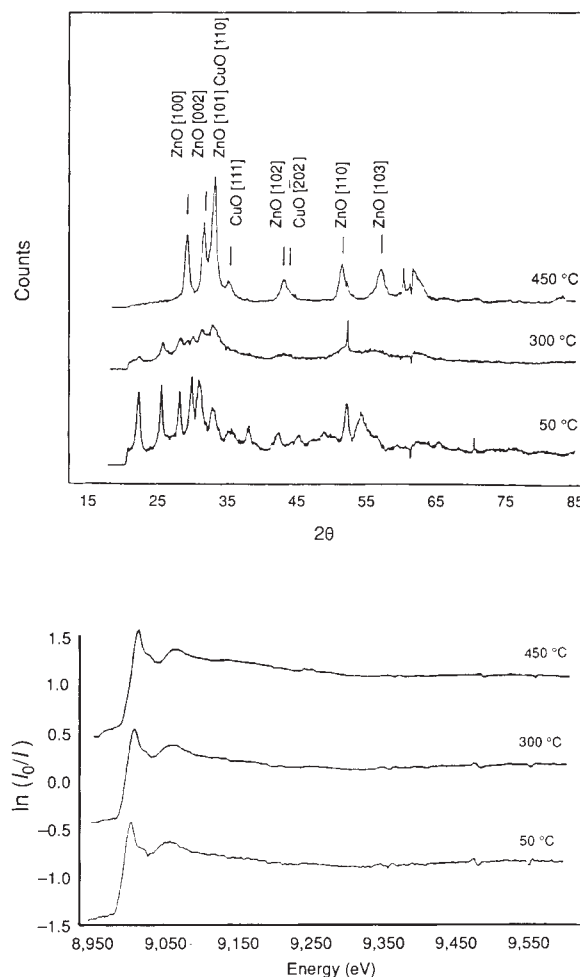


FIG. 2 Whereas marked changes in crystallinity (top) occur when aurichalcite is heated to  $450^\circ\text{C}$ , no significant changes occur in the local environment of  $\text{Cu}^{2+}$  ions when the original crystalline material becomes essentially amorphous (at  $\sim 300^\circ\text{C}$ ) and is then converted at ( $\sim 450^\circ\text{C}$ ) to a mixture of CuO and ZnO.



Energy calibration of the photodiode array was done using the X-ray absorption thresholds of 5- $\mu\text{m}$  foils of copper and zinc. Once calibrated, the array was used to determine the energy of the monochromatic beam used for the powder diffraction experiments. The  $2\theta$  calibration of the position-sensitive detector was determined using the reflections of a 200- $\mu\text{m}$  aluminium foil, based on the face-centred-cubic (f.c.c.) structure with a unit cell parameter of 4.0497 Å.

A typical series of combined absorption spectra and diffraction patterns is shown in Fig. 2 for a synthetic aurichalcite (Cu/Zn = 20.59/78.4) heated in air and ramped at  $2^\circ\text{C min}^{-1}$  from room temperature. The Cu K-edge and diffraction patterns (at a wavelength of 1.3885 Å) reveal that there is essentially no change in the immediate environment of the copper ions when the solid loses crystallographic order (at  $\sim 310^\circ\text{C}$ ), accompanying the breakdown of the carbonate and hydroxyl ions. Furthermore, there is no perceptible change in the environment of the  $\text{Cu}^{2+}$  ions when the decomposed aurichalcite ultimately crystallizes (at  $\sim 450^\circ\text{C}$ ) into a mixture of CuO and ZnO. By using a

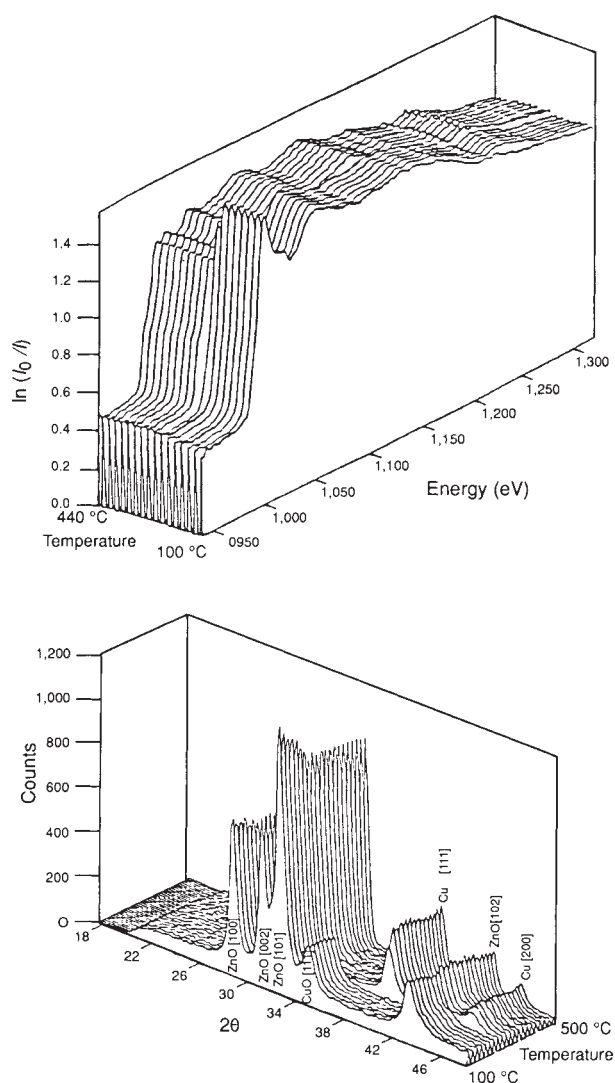


FIG. 3 *In situ* combined absorption-diffraction measurements recorded during the thermal reduction of 20:80 CuO:ZnO mixed oxides in a 10:90  $\text{H}_2$ : $\text{N}_2$  mixture with a heating rate of  $2^\circ\text{C min}^{-1}$ . The diffraction lines of metallic copper are indicated, but for clarity, the CuO [110], [200] and [111] reflections which are obscured by the [100], [002] and [101] reflections of ZnO, have not been labelled. Note the distinct changes in the pre-edge, near-edge and extended-edge fine structure of the absorption spectrum as the CuO is converted to copper at  $\sim 270^\circ\text{C}$ .

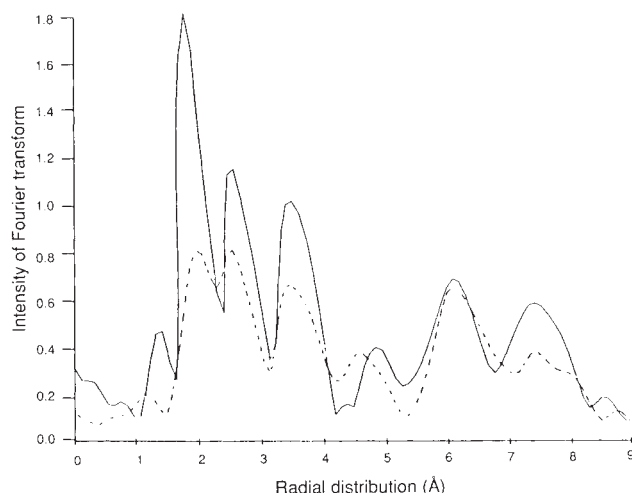


FIG. 4 Fourier transform plots (not corrected for phase shift) of the reduced mixed oxides at  $270^\circ\text{C}$  (solid line) and  $450^\circ\text{C}$  (dashed line).

wider bandpass so as to include the Zn K-edge in the absorption spectra, we also found that no discernible environmental changes occur around the  $\text{Zn}^{2+}$  ions on calcination of the as-prepared aurichalcite.

When the cooled, previously calcined product was heated afresh in a mixture of 10%  $\text{H}_2$  in  $\text{N}_2$  the CuO peaks were lost and metallic copper appeared at  $\sim 270^\circ\text{C}$ . Concomitantly, several well-defined changes occurred in the absorption spectra (Fig. 3). In particular, the initial resonances typical of CuO were replaced by the familiar two-peak threshold of metallic copper. There was also a chemical shift in the threshold to lower energies, and substantial changes in the absorption fine structure beyond the edge, with evidence of the oscillations characteristic of a f.c.c. metal. (No significant changes were observed in the near-edge or extended-edge beyond the Zn K-level absorption.) Estimates from line broadening indicate an average particle size for the Cu(0) aggregates of  $\sim 100$  Å. When the temperature of the reduced sample was increased to  $500^\circ\text{C}$  in the  $\text{H}_2$ - $\text{N}_2$  mixture, the line width of the copper metal narrowed further until it became limited by the response of the detector. Fourier transform analysis of oscillations in the extended X-ray fine structure (EXAFS) of the reduced samples (Fig. 4) showed a diminution in the first peak (corresponding to a Cu-Cu distance of 2.56 Å) at the higher temperature. We tentatively attribute this diminution to the static disorder resulting from incorporation of some metallic zinc into the metallic copper, forming a dilute, disordered phase of brass. This interpretation is in line with the observed 0.9% expansion in the unit cell parameter of copper metal at  $500^\circ\text{C}$ . We could detect no change in the cell parameter of the ZnO on proceeding from the mixed oxides to the catalytically active (supported copper) stage. We find no evidence, therefore, that our copper/zinc oxide catalyst contains some Cu(I) incorporated into the ZnO, as reported Kau *et al.*<sup>6,12</sup> for the methanol synthesis catalyst. But absence of evidence is not evidence of absence.

In separate experiments (D.W., in preparation, and unpublished work) on the catalytic performance of the Cu/ZnO samples derived from aurichalcite treated in this way, we observed a correlation between the surface area of copper metal (ranging from 7.4 to  $14.0\text{ m}^2\text{ g}^{-1}$ ) and catalytic activity in the reverse water-gas shift reaction at a temperature of  $240^\circ\text{C}$ . The mean turnover number is  $3.4 \times 10^{-4}\text{ CO}_2\text{ s}^{-1}\text{ Cu}(\text{surf})^{-1}$ . An *in situ* temperature-programmed-reaction spectroscopic analysis of the reverse water-gas shift reaction, carried out on a sample pre-reduced at  $260^\circ\text{C}$ , yielded turnover numbers for this

reaction which increased from  $8.43 \times 10^{-3}$  to  $5.15 \times 10^{-2} \text{ CO}_2 \text{ s}^{-1} \text{ Cu(surf)}^{-1}$  when the temperature rose from 340 °C to 400 °C. □

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## Environmental fine structure in low-energy $\beta$ -particle spectra

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SOME recent measurements of beta-decay spectra show anomalous line shapes which have been interpreted<sup>1–5</sup> as providing evidence for a neutrino with a mass of about 17 keV; other similar studies, however, find no anomalies<sup>6–13</sup>. Regardless of the reason for these discrepancies, such studies require a detailed understanding (at the level of accuracy of one part per thousand) of the spectral shape and the detector response expected in the absence of any exotic phenomena. Influences on the beta decay process from outside the nucleus are of great importance in this regard, and the role of atomic screening corrections<sup>14–16</sup> and atomic and molecular effects on the spectrum near the end point<sup>17</sup> have been considered previously. These produce only smooth, monotonic distortions of the spectrum. Here I note the existence of an effect, previously overlooked, that results in an oscillatory structure in the beta spectrum. The effect is of sufficient magnitude to be relevant to searches for heavy neutrinos that involve tritium beta decay, and it may also provide a basis for studies of molecular or crystal structure, in a manner analogous to extended X-ray absorption fine structure (EXAFS).

The physical mechanism I consider is similar to that producing the EXAFS observed above photo-absorption edges<sup>18,19</sup>. In that phenomenon, the ejected electron can be reflected from neighbouring atoms to produce a 'standing wave' in the region surrounding the absorbing atom. This gives rise to oscillations in the energy-dependent photo-absorption cross-section whose period depends on the interatomic distance and whose amplitude depends on the electron-atom scattering cross-section. There will be analogous oscillations in the spectrum of electrons emitted by the  $\beta$ -decay of an atom embedded in a molecule or crystal (beta environmental fine structure, or BEFS).

The probability  $P(E)$  that an electron of energy  $E$  is produced by the allowed  $\beta$ -decay of an isolated atom is governed by the electron-neutrino phase space and the interaction of the electron with the emitting atom<sup>20</sup>. The BEFS correction to this probability,  $\Delta P(E)$ , can be derived in analogy to the development of a

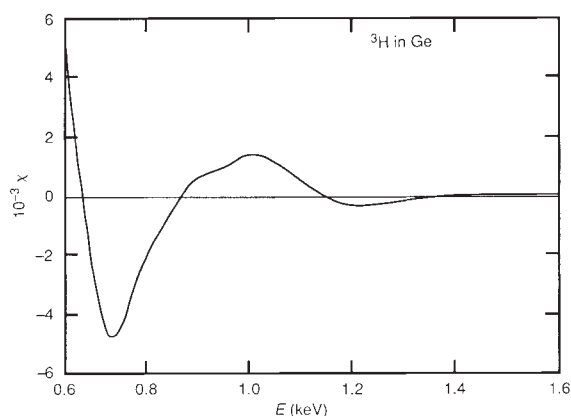


FIG. 1 BEFS expected for tritium in a germanium crystal at a temperature of 80 K. The tritium is assumed to occupy an interbond site 0.86 Å from one of the germanium atoms.

description of EXAFS<sup>19,21,22</sup>. In particular, the fractional BEFS modulation is

$$\chi(E) \equiv \frac{\Delta P}{P} = \sum_i \frac{|f_i(\pi)|}{kR_i^2} \sin(2kR_i + \phi_i + 2\delta_0) e^{-2R_i/\lambda} e^{-2\sigma_i^2 k^2} \quad (1)$$

Here, the sum is over all surrounding atoms (labelled by  $i$ ),  $f_i(\pi)$  is the amplitude for an electron of energy  $E = \hbar^2 k^2 / 2m$  to backscatter from atom  $i$ ,  $\phi_i = \arg f_i(\pi)$ ,  $\delta_0$  is the  $s$ -wave phase shift induced by the decaying (central) atom,  $\lambda(k)$  is the energy-dependent electron mean-free path, and  $\sigma_i$  is the root-mean-square thermal and zero-point fluctuation in  $R_i$ , the distance from the decaying nucleus to atom  $i$ . This expression, which is valid in the limit  $kR_i \gg 1$ , is very similar to that used to describe K-shell EXAFS<sup>19,23</sup>; the only differences are an overall sign and the appearance of the  $l=0$  rather than  $l=1$  central atom phase shift.

As a first illustration of the magnitude of BEFS expected, I consider the  $\beta$ -decay of tritium implanted in a germanium crystal, as was studied in ref. 2. The BEFS depends upon the precise atomic environment in which the tritium resides, but there is little direct information on this point. Muon-decay experiments in the analogous silicon crystal suggest that hydrogen occupies an interbond site 0.9 Å from the nearest silicon atom<sup>24</sup>. This distance can be interpreted as the difference between the Si-H bond distance in silane (1.48 Å) and the Si-Si bond distance (2.35 Å). I assume that a similar situation holds in germanium (germane bond distance of 1.59 Å and Ge-Ge bond distance of 2.45 Å), implying that tritium occupies an interbond site with one, one and three germanium atoms at distances of 0.86, 1.59 and 3.19 Å, respectively; all germanium atoms more distant than these can be neglected. For the nearest two atoms, I take  $\sigma = 0.073$  Å (corresponding to the zero-point motion of the Ge-T bond with frequency 1,059 cm<sup>-1</sup>) and for the latter three atoms, I take  $\sigma = 0.085$  Å, obtained by adding in quadrature the empirical Ge-Ge distance fluctuations at 80 K (refs 25, 26) and the Ge-T bond fluctuations. I adopt an energy-independent electron mean-free path of  $\lambda = 10$  Å, as suggested by these same EXAFS studies. The backscattering amplitude for electrons from germanium atoms is taken from a partial wave solution of the  $Z=32$  Lenz-Jensen potential<sup>27</sup>, and the central atom  $s$ -wave phase shift is that of the  $Z=2$  version of the same potential. My results for these electron-atom scattering quantities agree well with those given in ref. 28 at energies where they can be compared.



I show in Fig. 1 the resultant  $\chi(E)$  for  $\beta$  energies between 0.6 and 1.6 keV, the region important in experiments searching for a 17-keV neutrino. The BEFS pattern at these energies (where  $kR > 9$ ) is dominated by the contribution from the nearest germanium atom. It decreases strongly with increasing energy because of the Debye-Waller factor  $\exp(-2\sigma^2 k^2)$ , but is  $\sim 10^{-3}$  for energies below 1 keV. The roughly linear decrease of  $\phi$  and  $\delta_0$  with  $k$  expands the oscillations so that their period corresponds to an apparent radius of only 0.77 Å (roughly 0.07 Å of the 0.09 Å shift is due to  $\phi$  and 0.02 Å is due to  $\delta_0$ ). Contributions from the more distant atoms result in a slight anharmonicity at lower energies.

In Fig. 2 I show the BEFS expected for the decay of  $^{14}\text{C}$  in a germanium crystal, as was studied in ref. 5. I have assumed that the carbon atom substitutes for one of the germanium atoms without distorting the diamond-structure lattice. There are thus four, twelve and twelve neighbouring germanium atoms at distances of 2.45, 4.00 and 4.68 Å, respectively. I take the fluctuations for the first two shells of atoms to be  $\sigma = 0.058$  and 0.079 Å, respectively, as is obtained by scaling the EXAFS values for a germanium crystal at 80 K (refs 25, 26) by the square root of the ratio of the Ge-Ge to Ge-C reduced masses. As in the tritium case, the pattern is dominated by the nearest germanium atoms and is greater than  $10^{-3}$  for energies below about 1.1 keV; the larger distances involved result in more rapid oscillations. It should be noted that the BEFS patterns for both the tritium and carbon cases become more complex and larger for energies below those shown in the figures, approaching several per cent in amplitude at energies below 300 eV.

As a final illustration I consider molecular tritium in its vibrational ground state, as was studied in ref. 13. Here,  $R = 0.74$  Å and  $\sigma = 0.048$  Å (corresponding to a vibrational frequency of  $2,400\text{ cm}^{-1}$ ). I ignore electron screening entirely and put  $\lambda = \infty$ . I take the backscattering amplitude to be that of the  $Z = 1$  coulomb potential, so that  $\phi = 2\sigma_0$ , with  $\sigma_0$  the  $s$ -wave coulomb phase shift. In a similar spirit, I take the central atom phase shift to be that for the  $Z = 2$  unscreened coulomb field, augmented by a term associated with the asymptotic behaviour of the coulomb wavefunction that varies logarithmically in the internuclear distance. The BEFS pattern that results is shown in Fig. 3. The amplitude is only  $\sim 10\%$  of the previous two cases because of the smaller  $Z = 1$  backscattering amplitude relative to that of germanium and the smaller distance expands the oscillations. The data in ref. 13 are too imprecise by a factor of 2–4 to see the BEFS.

Although each of the above estimates could be refined (for example by including multiple scattering and by using improved

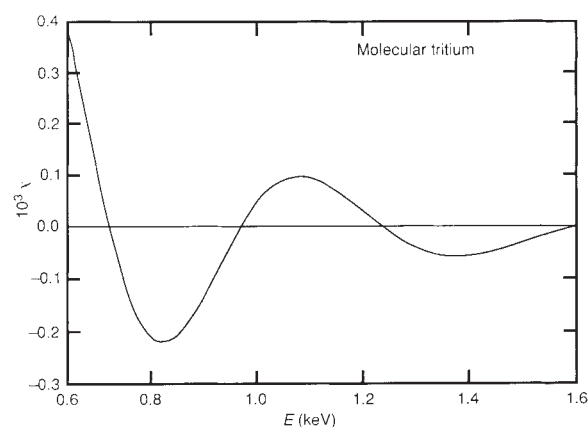


FIG. 3 The BEFS expected from the ground vibrational state of molecular tritium.

electron-atom potentials and an energy-dependent electron mean-free path), it should be clear from these calculations that BEFS is of a magnitude that could be measured. A search for the effect would be best carried out with a relatively heavy source atom (to minimize fluctuations) surrounded by high- $Z$  atoms (to maximize the back-scattering amplitude). A cooled source would also be advantageous, as the fluctuations in interatomic distances increase with temperature and wash out the BEFS pattern.

With regard to neutrino experiments, BEFS must be accounted for in searches for 17-keV neutrinos that use the tritium decay. At the  $10^{-3}$  level of accuracy currently of interest, it will be important only for those sources in which tritium is embedded in a host material. In these cases, it is obvious that attention must be paid to the chemical nature and temperature of the source, as well as to the effects of detector efficiency and resolution on the observed BEFS pattern; the pattern for germanium could differ in detail from that shown in Fig. 1 if my assumption about the crystallographic site of hydrogen is incorrect. Although it is unlikely that BEFS could be the origin of all of the reported anomalies, it could change the interpretation of these experiments. Note also that BEFS is unimportant in neutrino mass experiments that use higher-energy ( $\sim 150$ -keV) electrons, as it is suppressed to negligible levels by the Debye-Waller factors.

More generally, BEFS might be used as a kind of 'internal EXAFS' for structural studies. For example, the precise shape and magnitude of the BEFS for tritium in germanium silicon crystal would provide information on the site of hydrogen in semiconductors. Another possibility is the potential for radioisotope labelling to allow BEFS studies of specific sites in large molecules. The relatively small amounts of material required, the circumvention of a large synchrotron light source, and the present sophistication in the interpretation of EXAFS will probably encourage such studies. □

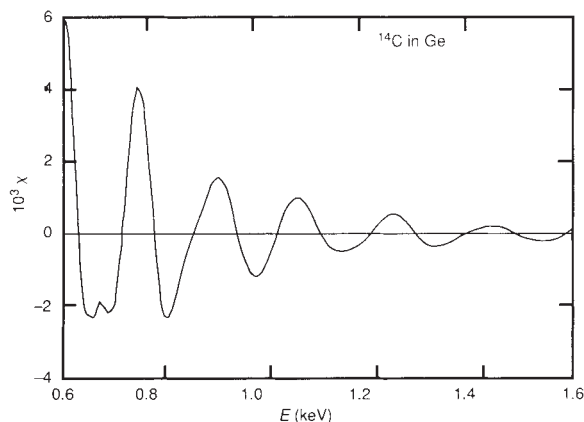


FIG. 2 Similar to Fig. 1 for a  $^{14}\text{C}$  atom in germanium crystal. The carbon atom is assumed to substitute for a germanium atom in the diamond-structure lattice without distortion.

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## Microstructure of isotropic materials with negative Poisson's ratio

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**MATERIALS** that expand in the transverse direction under uniaxial extension, or that contract laterally when compressed, are said to have a negative Poisson's ratio,  $\nu$ . For an isotropic elastic material,  $\nu$  is the negative of the ratio of lateral to axial strain under uniaxial extension or compression. Despite the apparently counter-intuitive nature of this behaviour,  $\nu < 0$  has been observed for some anisotropic crystals<sup>1</sup> and materials comprised of fibrous networks<sup>2,3</sup>, when loaded in a specific direction. Lakes<sup>4</sup> has described a class of foams that constitute perhaps the only known isotropic materials with negative  $\nu$ . Materials of this sort are expected to have interesting mechanical properties, such as high energy absorption and fracture resistance, which may be useful in some applications<sup>4</sup>. Here we describe a general class of microstructures that lead to a negative Poisson's ratio, and show that some existing and hypothetical materials with this property share features common to this class. Our microstructural model provides insight into why natural materials of this kind are rare, and suggests a general methodology for designing such materials.

Theoretical bounds on Poisson's ratio can be obtained by requiring that the strain energy of an elastic isotropic solid remains positive under any set of deformations. This gives the bounds  $-1 \leq \nu \leq \frac{1}{2}$ . The upper limit corresponds to materials that cannot change volume, and the lower limit applies to materials that cannot change shape.

The first attempt to relate  $\nu$  to a specific microstructure belongs to Poisson himself, who considered a material consisting of randomly packed smooth spherical particles interacting by forces that are directed along the line connecting particle centres. For this system he determined  $\nu = \frac{1}{4}$ .

Real granular materials have frictional interfaces and can transmit forces that have a component tangential to the plane of contact. To account for shear resistance at contacts it is reasonable to assume that the tangential force  $f_t$  acting at a contact is proportional to the relative tangential displacement  $\delta_t$  at a contact point:  $f_t = k_t \delta_t$ , where  $k_t$  is the tangential contact

stiffness and determines resistance of a contact to shear displacement between two particles. Similarly, for a force  $f_n$  and displacement  $\delta_n$  normal to the plane of contact, we assume  $f_n = k_n \delta_n$ , where  $k_n$  is the normal stiffness which defines resistance of a contact to compression directed along the line connecting centres of two spherical particles. The Poisson's ratio of such a system can be expressed as follows<sup>5</sup>

$$\nu = \frac{1 - \lambda}{4 + \lambda} \quad (1)$$

Here  $\lambda$  is the ratio of tangential to normal contact stiffness. For smooth spheres with no shear resistance at contacts,  $\lambda = 0$ , giving Poisson's result,  $\nu = \frac{1}{4}$ . An important feature of equation (1) is that  $\nu$  is negative for  $\lambda > 1$  (tangential contact stiffness is greater than normal contact stiffness). For granular materials, however,  $\nu > 0$ .

This description is equally applicable to materials that can

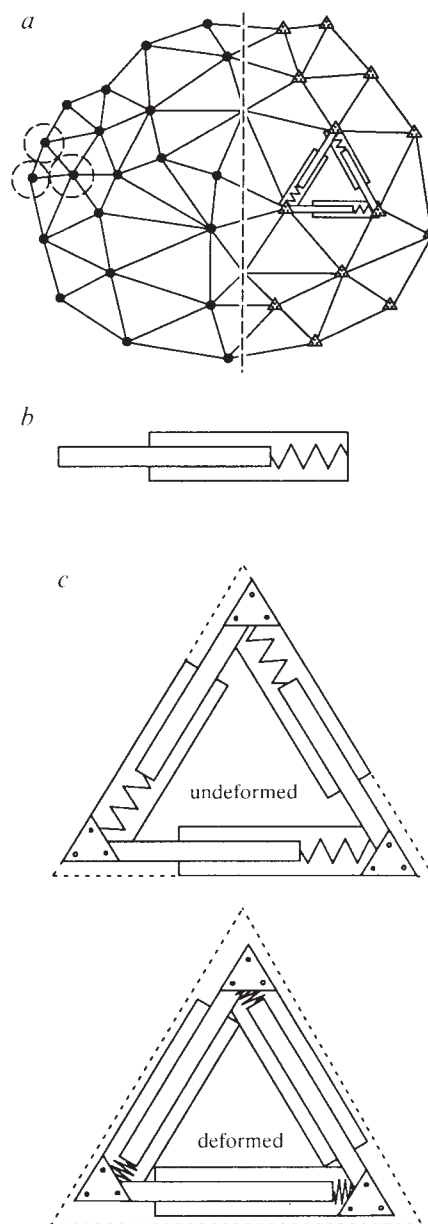


FIG. 1 Simple truss structure leading to negative Poisson's ratio behaviour. a, Network of elastic elements; b, shock absorber unit; c, triangular cell unit.



be modelled by networks of randomly oriented elastic elements, as shown in Fig. 1a. The connection between elastic networks and granular materials becomes evident if nodes of the network are associated with particle centres, and forces acting at intergranular contacts are identified with forces in network elements (left side of Fig. 1a). Networks consisting of numerous truss-like elements respond to deformations similarly to isotropic elastic materials if elements of all orientations are encountered with equal probability<sup>5</sup>. Each element of such a network can be characterized by stiffness in the axial direction,  $k_n$  and in the transverse direction,  $k_t$ .

To understand the origin of the negative Poisson's ratio effect qualitatively, it is convenient to consider plane networks in which deformation mechanisms can be visualized. Poisson's ratio of plane networks is also related to  $\lambda = k_t/k_n$  as follows<sup>5</sup>

$$\nu = \frac{1 - \lambda}{3 + \lambda} \quad (2)$$

As previously, Poisson's ratio of plane networks is negative for  $\lambda > 1$ . When the shear stiffness of contacts is much greater than the normal stiffness ( $\lambda \rightarrow \infty$ ),  $\nu \rightarrow -1$ .

If the network consists of rectangular bars then  $\lambda < 1$ , and  $\nu$  is positive. To explore the influence of high tangential stiffness, consider the plane structure constructed from elements analogous to shock absorbers (Fig. 1b). These are characterized by a very low stiffness in axial compression or tension and a high shear stiffness. If three such elements are linked to form a triangular structure (Fig. 1c), then the resulting group is deformable but the deformations are highly constrained, as the internal angles of the triangle must be preserved. Consequently, if the group is compressed in one direction it will contract overall.

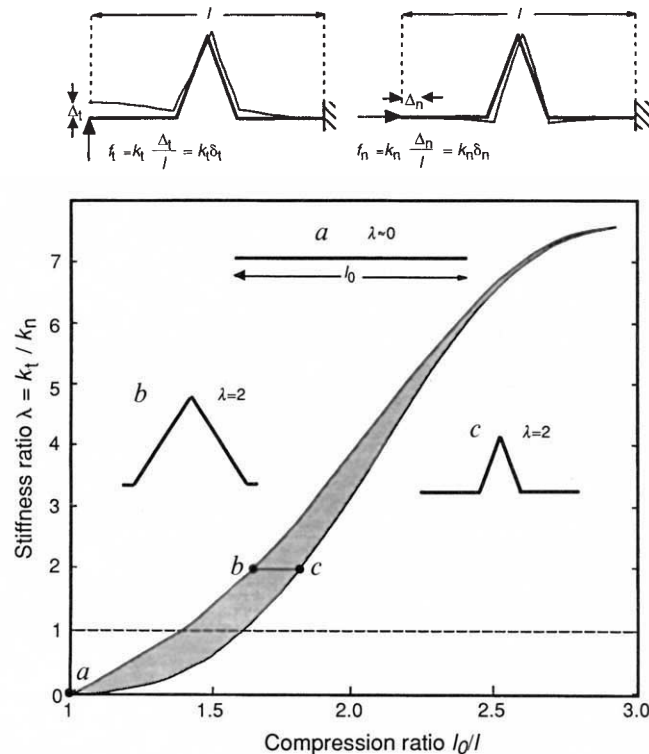


FIG. 2 Stiffness ratio of compressed bar elements. The upper diagrams illustrate tangential (left-hand side) and normal (right-hand side) compression of deformed bar elements; the thinner line shows the shape after compression. Insets *a*, *b* and *c* show the bar shape corresponding to points *a*, *b* and *c* on the curve; *b* and *c* have the same compression ratio, but their different shapes, and in particular their different kink angles, lead to different values of  $\lambda$ .

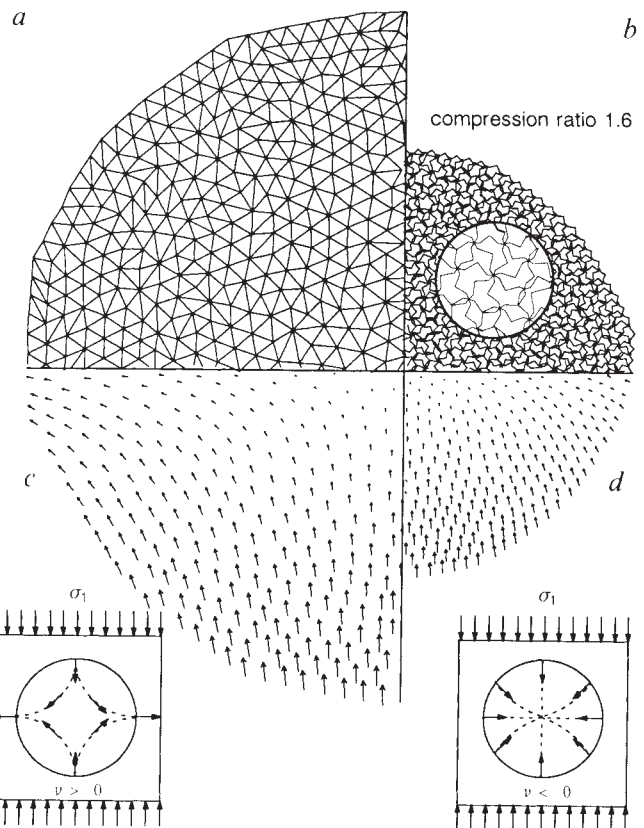


FIG. 3 *a*, Original network; *b*, compressed network; *c*, node trajectories in compression test of original network; *d*, node trajectories in compression test of compressed network.

The same applies for uniaxial extension. A random structure consisting of triangular elements can be made isotropic if shock absorber elements of all orientations are present in the system with equal probability. This can always be achieved for a network of elements with randomly varying length.

During deformations, all triangles will remain similar and the system deforms as if drawn to a different scale (Fig. 1c). Because strains in all directions are equal and have the same sign,  $\nu$  for the system is  $-1$ . The essential part of this explanation is preservation of angles between truss elements because of high shear stiffness. This is the physical interpretation of mechanisms that lead to negative Poisson's ratio according to equations (1) and (2). An example of an isotropic system with  $\nu = -1$  has been given previously<sup>7</sup>.

The above example relates to an artificial system in which high-shear-stiffness elements lead to  $\nu < 0$ . A natural system that can be modelled by three-dimensional networks of interconnected elements joined at nodes is open-cell foam<sup>8</sup>. Deformation of foams in the elastic range is dominated by bending of bar elements. At large compressive deformations under elevated temperature, plastic collapse of foam cells takes place, and the foam shows a negative Poisson's ratio<sup>4</sup>.

One way of visualizing the structure of a collapsed foam is to consider a network in which collapsed bar elements are deformed permanently and protrude inwards (Fig. 2). This type of microstructure was described by Lakes<sup>4</sup> as 'reentrant'. The axial stiffness of the collapsed bar is greatly reduced because the element is curved; it is primarily determined by the kink angle. Figure 2 illustrates ranges of  $\lambda = k_t/k_n$  obtained for different bar compression ratios. The range corresponds to different configurations of element shapes that can be obtained for the same compression ratio. According to the above theory,

compression ratios in excess of 1.5 correspond to  $\lambda > 1$  and should result in materials with negative Poisson's ratio determined by formulae (1) and (2).

To check theoretical conclusions, we carried out a computer simulation of two plane networks. The first network consisted of straight elastic bar elements, the second consisted of compressed elements with a kink angle corresponding to a compression ratio of  $\sim 1.6$  ( $\lambda = 2$ ). The original and the compressed network are shown in Fig. 3a and b. Both networks were compressed uniaxially. Figure 3c and d illustrate the results of these simulations by showing trajectories of network nodes under uniaxial compression. The original network has  $\nu = 0.33$ , whereas the compressed network has  $\nu = -0.25$ , as determined by equation (2).

The generality of the theory can be tested by applying it to a class of molecular systems. Wojciechowski<sup>9</sup> suggests that solids consisting of nonconvex hexagon-type molecules (hexamers) may have negative Poisson's ratio. Such molecules can be modelled in two dimensions by rigidly connected discs ('atoms') at the vertices of a hexagon (Fig. 4a). If discs are assumed to be soft at the exterior and have a hard elastic core, neighbouring molecules can interpenetrate (Fig. 4b). Wojciechowski<sup>9</sup> has shown, both theoretically and using numerical simulations, that such a system has negative Poisson's ratio when interpenetration of particles is large. As hexamer particles are roughly circular, their interactions are not far different from interactions between sand particles. Although the contact cannot be treated as a point, there is still a force acting between particles, with a component  $f_n$  along a line connecting particle centres as well as a component  $f_t$  acting perpendicular to this line (Fig. 4c). Both force com-

ponents cause displacements in their respective directions, as in the case of sand particles. If the core of hexamer 'atoms' is assumed elastic, normal and tangential force components will be proportional to the corresponding displacements, and the parameter  $\lambda = k_n/k_t$  can be calculated. Figure 4c illustrates the dependence of  $\lambda$  on the interpenetration of molecules, expressed in terms of a ratio of the original cell dimension to the reduced cell size. Different cell geometries are modelled by changing the diameter of the elastic core while preserving the distance between vertices of the perimeter molecules.

Interpenetration of the hexamer particles reduces the normal stiffness whereas the tangential stiffness increases. When the diameter of the elastic core is 0.7 of the particle diameter, both stiffness components become equal ( $\lambda = 1$ ) and  $\nu = 0$ . For larger interpenetrations, Poisson's ratio becomes negative. Wojciechowski<sup>9</sup>, using a completely different analysis, reaches virtually identical conclusions.

Strictly, the analysed hexamer system is an anisotropic crystal with a 6-fold symmetry. Similar systems are known to behave as isotropic solids as far as deformation properties are concerned. It is not difficult to construct a truly isotropic system with the same type of behaviour. Any assembly of particles with nonconvex surfaces that allow sufficient interpenetration should show a negative Poisson's ratio. The main cause of this unusual behaviour is greater stiffness of microstructural elements in shear than in compression. □

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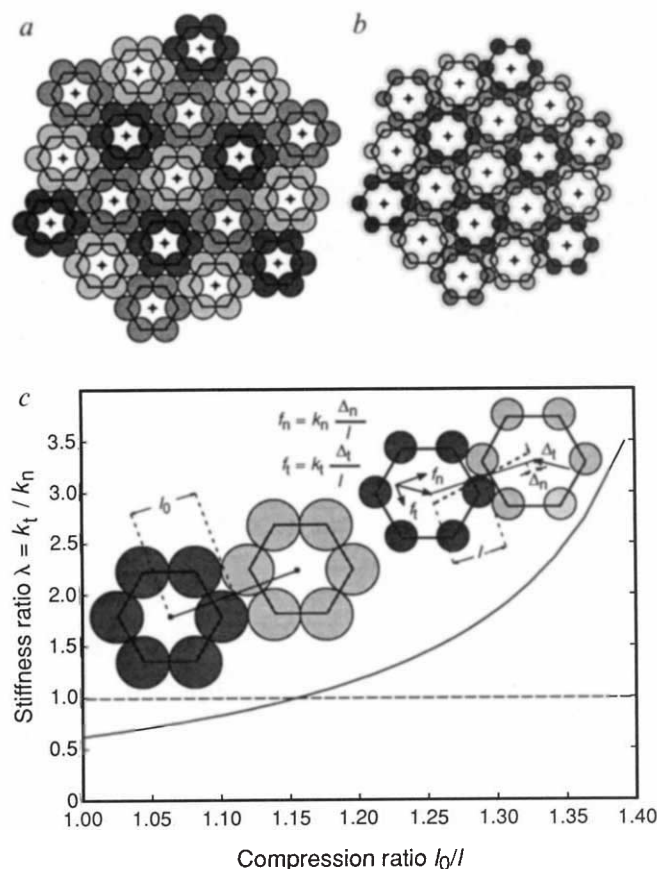


FIG. 4 a, Two-dimensional hexagonal array of rigidly connected discs. b, Interpenetration of these discs, assumed to be soft at the outside and to have a hard elastic core. Individual 'molecules' are indicated by the shading. c, Stiffness ratio as a function of compression ratio for the array of discs. Poisson's ratio becomes negative when  $\lambda > 1$ , which occurs at high interpenetration.

## Organic matter and containment of uranium and fissionogenic isotopes at the Oklo natural reactors

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SOME of the Precambrian natural fission reactors at Oklo in Gabon contain abundant organic matter<sup>1,2</sup>, part of which was liquefied at the time of criticality and subsequently converted to a graphitic solid<sup>3,4</sup>. The liquid organic matter helps to reduce U(VI) to U(IV) from aqueous solutions, resulting in the precipitation of uraninite<sup>5</sup>. It is known that in the prevailing reactor environments, precipitated uraninite grains incorporated fission products. We report here observations which show that these



uraninite crystals were held immobile within the re-solidified, graphitic bitumen. Unlike water-soluble (humic) organic matter, the graphitic bituminous organics at Oklo thus enhanced radionuclide containment. Uraninite encased in solid graphitic matter in the organic-rich reactor zones lost virtually no fissionogenic lanthanide isotopes. The first major episode of uranium and lead migration was caused by the intrusion of a swarm of adjacent dolerite dykes about 1,100 Myr after the reactors went critical. Our results from Oklo imply that the use of organic, hydrophobic solids such as graphitic bitumen as a means of immobilizing radionuclides in pretreated nuclear waste warrants further investigation.

Small pockets of uranium ore at Oklo went critical  $1,950 \pm 30$  Myr ago<sup>6</sup>, producing several natural fission reactors. Reactors 1–6 contain virtually no organic matter. Total energy production in these reactors was 16,500 MW yr. Roughly 6 tons of  $^{235}\text{U}$  fissioned; the total uranium content was 800 tons. Reactors 7–9 contain abundant organic matter with heterogeneous distributions. They produced 1,300 MW yr of energy, and 480 kg of  $^{235}\text{U}$  fissioned; their total uranium content<sup>6</sup> was 80 tons. The reactors are located in the C-1 stratum of the Proterozoic Franciscan Series, which consists of sedimentary rocks<sup>7,8</sup>. Nuclear fission reactions occurred in ores with 25–60% uranium. Water acted as a moderator; the reactions ceased after  $10^5$ – $10^6$  years (ref. 9) when too little fissile material remained. Temperatures during criticality ranged between 160 and 360 °C in the reactors<sup>7,9</sup>. Organic matter in reactors 7–9 consists of kerogen and now solid bitumen<sup>4,10,11</sup>.

Organic matter in the Oklo natural reactors 7–9 could have important effects on radionuclide containment. This assumption is based on observations at other uranium deposits<sup>12</sup>, where solid organic matter prevented the movement of uraninite, uranium and radiogenic products. Although liquid bitumen was generated by hydrothermal solutions from the uraniferous organic solid in these deposits<sup>12</sup>, migration and leaching of uraninite and radionuclide mobilization by bitumen were minimal. Our aim was therefore to determine whether the same occurred at Oklo and, if it did, whether one could thereby learn lessons for nuclear waste storage.

The following sequence of events is known to have occurred in radionuclide containment at Oklo. First, liquid bitumen was derived from syngenetic kerogen through hydrothermal processes<sup>13</sup> at the time of criticality<sup>14</sup>. Second, the abundant liquid bitumen (and less-available kerogen) reduced U(VI) from aqueous solutions (possibly by  $\pi$ -electron, redox atom transfer and/or radiation-induced reduction, each involving organics). This resulted in the precipitation of uraninite, in which fission products were incorporated. Next, liquid bitumen was intruded into fractures and pores<sup>3</sup> during and after criticality. Finally, bitumen solidified at these sites, immobilizing the enclosed uraninite. Heat soon turns bitumen into a solid containing cryptocrystalline graphite<sup>15</sup>.

Migration of liquid bitumen in the reactors and surrounding rocks is revealed by organic petrography, which shows narrow (micrometre-sized) and wider (millimetre or centimetre-sized) fractures, intruded by and filled with bitumen<sup>2</sup>, as well as granular bitumen filling the pore and void spaces in rocks (Table 1, column 3). The non-mobilized organic matter is kerogen, which is a syngenetic solid<sup>14</sup>. Electron microprobe analyses showed that kerogen and bitumen in and near the reactors (at least 200 m away) contains mainly micrometre-sized dispersed uraninite. Bitumen also contains crystals of galena which represent, in part, precipitated radiogenic lead. Distant organic samples contain no uraniferous minerals. Reflected light microscopy showed textural features indicating that uraninite was not effectively mobilized by bitumen, which became increasingly viscous and then solidified before much migration and transport of uraninite could occur. Solidification and graphitization of bitumen were caused by the heat in the reactors (the temperature varied in space and time).

TABLE 1 Constituents of organic-rich sedimentary rocks in and near Oklo natural fission reactors

| Sample number | Sample location                                             | Organic constituents                                            | Kerogen<br>$R_{0,\text{max}}(\%)$ , $R_{0,\text{min}}(\%)$<br>$\bar{R}_{0,\text{max}}(\%)$ |
|---------------|-------------------------------------------------------------|-----------------------------------------------------------------|--------------------------------------------------------------------------------------------|
| 1             | Reactor 7 within reactor core                               | Kerogen particles; granular bitumen                             | 3.58, 0.98<br>2.57 ( $n=47$ )                                                              |
| 2             | Reactor 9 at reactor core edge                              | Kerogen particles; bitumen in veins                             | 3.52, 1.23<br>2.22 ( $n=41$ )                                                              |
| 3             | At reactor core edge                                        | Galena crystal, isolated from organic matter                    | ND, inorganic phase                                                                        |
| 4             | At reactor core edge                                        | Kerogen in two populations; abundant bitumen                    | 3.24, 1.24<br>2.49 ( $n=38$ )                                                              |
| 5, 6          | 1.2 m from reactor core                                     | Granular bitumen; kerogen particles                             | ND                                                                                         |
| 7             | Reactor 8, 50 m from reactor core                           | ND                                                              | ND                                                                                         |
| 8, 9, 10      | Reactor 9, 200 m from reactor core                          | Bitumen in veins; kerogen particles                             | 4.54, 0.77<br>3.08 ( $n=36$ )                                                              |
| 11            | 5 km from the reactors at the Boyindzi U ore deposit        | Bitumen in veins; kerogen particles                             | 6.04, 0.89<br>3.93 ( $n=37$ )                                                              |
| 12            | 8 km from the reactors, from a vein traversed by drill core | Highly solidified, vitreous organic matter derived from bitumen | 5.16, 3.78<br>4.95 ( $n=25$ )                                                              |
| 13            | 50 km from the reactors, FB black shale                     | Abundant kerogen, 'coked' equivalent of bitumen                 | 5.18, 4.02<br>5.01 ( $n=5$ )                                                               |

ND, not determined. Reflectance measurements, in oil, were made on kerogen using a Zeiss Axioskop microscope under incident white and blue light, and a six-in-one standard ( $R_0=0.3$ – $1.68\%$ ).  $R_{0,\text{max}}$ ,  $R_{0,\text{min}}$  are maximum and minimum reflectances, measured in oil.  $\bar{R}_{0,\text{max}}$  is the mean maximum reflectance for  $n$  measured points. Gas chromatography mass spectrometry (discussed in the text) was carried out at 450 °C and 900 °C, with a Chemical Data Systems Pyroprobe coupled to a Hewlett-Packard 5710A gas chromatograph (25 m  $\times$  0.31 mm internal diameter, 5% phenyl methyl silicone, fused silica column) and a Finnegan MAT model 700 mass spectrometer with ion trap detector. Mineral constituents were identified with a JEOL-733 electron microprobe. Microfocused laser Raman spectra (discussed in the text) were obtained with a 25-W Ar laser, using 544-nm light, with 60–90 mW, transmitted to an Olympus BH2 microscope and to a Jobin Yvon model U spectrometer.

Migration of uraninite in bitumen has also been very limited at the 2,300-Myr-old Elliot Lake uranium deposits in Canada<sup>12</sup>. In bitumen, uraninite microcrystals were virtually unleached, and uranium, thorium, samarium and so on were insoluble and did not migrate<sup>12</sup>, just like at Oklo. But humic materials, which contain carboxyl and similar groups, readily form complexes with uranium and other actinide elements, and form colloids affecting radionuclide migration in groundwater<sup>16</sup>. Humic colloids can remain in suspension in water for a long time and are known to be transported over significant distances by groundwater<sup>17</sup>. Such radionuclide migration did not occur at Oklo. Solid bitumen in the Oklo reactors contained highly condensed aromatic hydrocarbons with abundant, dispersed cryptocrystalline graphite, but no detectable carboxyl groups<sup>3</sup>. Therefore, this solid Oklo organic substance did not form colloids, could not migrate and was not a vehicle for actinide transport. Thus, organic solids consisting of highly condensed aromatic hydrocarbons and abundant graphite effectively contain uraninite and radionuclides.

Bitumen and kerogen in and near the reactors have less graphitic and less tightly bound structures than distant organics. Uraninite and fissionogenic isotopes may be more easily released from these structures, enhancing radionuclide loss from the reactors; this possibility had to be tested. Microfocused-laser Raman spectra showed that the hydrocarbons in and around the reactors contain cryptocrystalline graphite of smaller particle sizes than organic matter at the distant (8, 50 km) locations. In and near the reactor cores, reflectances of organic matter are relatively low. At sites distant from the reactors, reflectances are high because graphitization is better developed and molecular condensation of aromatic hydrocarbons is more extensive (Table 1, column 4). Pyrolysis/gas-chromatography/mass spectrometry

TABLE 2 Isotope ratios and  $^{207}\text{Pb}/^{206}\text{Pb}$  ages of samples in and near the organic-rich Oklo natural fission reactors

Isotope ratios of U and Pb and Pb–Pb ages of organic matter containing minute dispersed minerals.

| Sample number | $^{235}\text{U}/^{238}\text{U}$ | U (p.p.m.) | Pb (p.p.m.) | $^{204}\text{Pb}/^{206}\text{Pb}^*$ | $^{207}\text{Pb}/^{206}\text{Pb}^*$ | $^{208}\text{Pb}/^{206}\text{Pb}^*$ | $^{206}\text{Pb}/^{238}\text{U}^\dagger$ | $^{207}\text{Pb}/^{235}\text{U}^\ddagger$ | $^{207}\text{Pb}/^{206}\text{Pb}^\ddagger$<br>Age (Myr) |
|---------------|---------------------------------|------------|-------------|-------------------------------------|-------------------------------------|-------------------------------------|------------------------------------------|-------------------------------------------|---------------------------------------------------------|
| 2‡            | 0.00725                         | 103,000    | 13,150      | 0.000128                            | 0.09684                             | 0.00633                             | 0.13458                                  | 1.7659                                    | 1,529                                                   |
| 3‡            | ND                              | 30         | 558,000     | 0.000153                            | 0.11138                             | 0.00783                             | ND                                       | ND                                        | 1,788                                                   |
| 4‡            | 0.00716                         | 4,150      | 7,910       | 0.000212                            | 0.12453                             | 0.01090                             | 1.9451                                   | 32.994                                    | 2,004                                                   |
| 5‡            | ND                              | <10        | 102         | 0.000730                            | 0.04579                             | 0.03160                             | ND                                       | ND                                        | –707                                                    |
| 6‡            | ND                              | 14         | 215         | 0.000417                            | 0.06464                             | 0.02433                             | ND                                       | ND                                        | 554                                                     |
| 7‡            | 0.00725§                        | 11,930     | 9,200       | 0.000169                            | 0.11477                             | 0.00858                             | 0.7975                                   | 12.437                                    | 1,851                                                   |
| 8§            | 0.00725                         | ND         | ND          | 0.000024                            | 0.06360                             | 0.00084                             | 0.0763                                   | 0.665                                     | 717                                                     |
|               | 0.00725                         | ND         | ND          | 0.000021                            | 0.06314                             | 0.00076                             | 0.0665                                   | 0.576                                     | 704                                                     |
|               | 0.00725                         | ND         | ND          | 0.000012                            | 0.06182                             | 0.00048                             | 0.0720                                   | 0.612                                     | 665                                                     |
|               | 0.00725                         | ND         | ND          | 0.000019                            | 0.06214                             | 0.00064                             | 0.0512                                   | 0.437                                     | 670                                                     |
|               | 0.00725                         | ND         | ND          | 0.000024                            | 0.06384                             | 0.00093                             | 0.0814                                   | 0.713                                     | 730                                                     |
|               | 0.00725                         | ND         | ND          | 0.000025                            | 0.06377                             | 0.00091                             | 0.0626                                   | 0.547                                     | 722                                                     |
|               | 0.00725                         | ND         | ND          | 0.000015                            | 0.06271                             | 0.00078                             | 0.0724                                   | 0.624                                     | 690                                                     |
|               | 0.00725                         | ND         | ND          | 0.000015                            | 0.06226                             | 0.00059                             | 0.0774                                   | 0.662                                     | 675                                                     |
| 9§            | 0.00725                         | ND         | ND          | 0.000015                            | 0.06300                             | 0.00068                             | 0.1026                                   | 0.887                                     | 700                                                     |
|               | 0.00725                         | ND         | ND          | 0.000020                            | 0.06270                             | 0.00059                             | 0.1002                                   | 0.860                                     | 683                                                     |
|               | 0.00725                         | ND         | ND          | 0.000018                            | 0.06350                             | 0.00066                             | 0.1006                                   | 0.876                                     | 715                                                     |
|               | 0.00725                         | ND         | ND          | 0.000014                            | 0.06443                             | 0.00058                             | 0.1091                                   | 0.964                                     | 745                                                     |
|               | 0.00725                         | ND         | ND          | 0.000025                            | 0.06450                             | 0.00099                             | 0.1026                                   | 0.906                                     | 742                                                     |

Isotope ratios of U, Mo, Ag, Sn, Te, Nd and Sm

| Sample number           | $^{235}\text{U}/^{238}\text{U}$ | Mo  <br>98/95 | Ag  <br>109/107 | Sn  <br>120/124 | Te  <br>128/130 | Nd§<br>144/143 | Sm§<br>149/147 |
|-------------------------|---------------------------------|---------------|-----------------|-----------------|-----------------|----------------|----------------|
| Natural isotopic ratios | 0.00725                         | 1.5           | 0.92            | 5.7             | 0.90            | 1.95           | 0.92           |
| 1                       | 0.00678¶                        | —             | 0.49#           | —               | 0.18#           | 1.14¶          | 0.03¶          |
| 2                       | 0.00725‡                        | 1.5           | —               | 6.0             | 0.40            | 1.27           | 0.45           |
| 4                       | 0.00716‡                        | 1.1           | 0.60            | 5.0             | 0.17            | —              | 0.12           |
| 5                       | —                               | 1.4           | —               | 6.0             | —               | —              | 0.90¶          |
| 7                       | 0.00725§                        | 1.5           | 0.60            | 5.2             | 0.14            | 1.84           | 0.78           |
| Fission product ratios  | —                               | 0.90          | 0.30            | 0.5             | 0.18            | 1.11           | <0.1           |

Decay constant  $\lambda_{^{238}\text{U}} = 0.15513 \times 10^{-9} \text{ yr}^{-1}$ ,  $\lambda_{^{235}\text{U}} = 0.98485 \times 10^{-9} \text{ yr}^{-1}$ ; normal  $^{235}\text{U}/^{238}\text{U} = 0.00725$ . ND, not determined. Fission product ratios (bottom of table) are theoretical and adjusted for published parent abundances<sup>18</sup> in reactor 9.

\* Corrected for fractionation and blank only.

† Corrected for fractionation, blank and common lead; age data were calculated using measured U isotopic compositions;  $2\sigma$  errors are 2–4 Myr.

‡ Lead and uranium isotopes measured with a VG354 thermal ionization mass spectrometer following dissolution of samples in a mixture of  $\text{HClO}_4$ ,  $\text{HNO}_3$  and HF, spiking of aliquots with  $^{205}\text{Pb}$  and  $^{235}\text{U}$  and standard column separation procedures (except where noted otherwise); samples were individual grains weighing 10–50  $\mu\text{g}$ .  $2\sigma$  error range is  $\pm 0.1$ –0.2% for all  $^{207}\text{Pb}/^{206}\text{Pb}$  and  $^{238}\text{U}/^{235}\text{U}$  ratios.

§ Determined with CAMECA IMS3f ion microprobe mass spectrometer on individual uraninite crystals<sup>23</sup>; typical spot analysed was 20  $\mu\text{m}$  in diameter.  $2\sigma$  error range is 0.1%, 0.5% and 1.0% for  $^{207}\text{Pb}/^{206}\text{Pb}$ ,  $^{206}\text{Pb}/^{238}\text{U}$  and  $^{207}\text{Pb}/^{235}\text{U}$  ratios, respectively, with a precision of 0.08% after correction for mass discrimination effect (0.2% per a.m.u.). Accuracies of U, Nd, and Sm isotope ratios are  $\pm 0.02$ ,  $\pm 0.02$  and  $\pm 0.01$ , respectively.

|| Measured with VG PQII inductively coupled plasma mass spectrometer in argon, after laser ablation of polished surfaces using a Spectron neodymium–yttrium–aluminium garnet laser to volatilize millimetre-sized areas of organic samples. Accuracy of the isotope ratios measured by this mass spectrometer is  $\pm 0.1$  for Mo and Ag, and  $\pm 1$  for Sn and Te.

¶ Analyses done on 10-mg powdered sample by thermal ionization mass spectrometry. Results permit calculation of the apparent age of fission reaction:  $\text{age} = 1/\lambda_{^{235}\text{U}} \ln(A_f/N_f/U_f)$ , where  $\lambda_{^{235}\text{U}}$  is the radioactive decay constant of  $^{235}\text{U}$ ,  $N_f/U_f$  is the number of fissions of  $^{235}\text{U}$  relative to present-day U; the abundance of fissionogenic Nd is directly related to the  $^{235}\text{U}$  content involved in the fission reactions and is used to define the value of  $N_f$ .  $\tau$  is the fluence received by the sample, and  $A$  is a factor of nuclear parameters of samples and spectrum index<sup>26</sup>. The mean Nd age of three samples at reactor core 9 is 1,300 Myr; the age of sample 1 within reactor 7 is  $1,850 \pm 15$  Myr.

# Calculated from ref. 28.

confirmed this trend. The thermal energy imparted by the pyrolysis experiment was less capable of fragmenting bitumen and kerogen structures in distant samples than the less tightly bound organic solids in and around the reactors. Hydrothermal reactions and radiation, leading to addition of H,  $\text{H}_2$  and so forth, and neutron fluence are the most likely explanation for the less tightly bound organic structures at the reactors. Uraninite crystals remained immobile within this solid organic matter. Microscopic observations revealed no evidence that hydrothermal effects and radiation damage dislodged uraninite from the graphitic bitumen and kerogen in and around the reactors.

Ion microprobe mass spectrometry showed that several fission products were incorporated in this uraninite when it precipitated at the edge of the reactor cores (Table 2). Molybdenum and tin show no abnormal isotopic compositions, whereas silver, tellurium, neodymium and samarium have large fissionogenic contributions even at a distance of 50 m from the reactor core (Table 2, sample 7). This is in agreement with previous findings<sup>18</sup> in the peripheral rocks of reactor 9. Migration of fission products

to limited distances from the reactor core was possibly caused during criticality by convective aqueous solutions, before hot water could generate enough bitumen to precipitate uraninite, which incorporates fission products. Reactor 9 contains uraninite both enclosed in organic matter (from which nuclides were not lost) and enclosed in clays (from which neodymium was lost). As much as 30% of fissionogenic neodymium, relative to uranium, has been lost by those samples<sup>19,20</sup> that are most depleted in  $^{235}\text{U}$ , in reactor core 9. Similarly, restricted local migration of lanthanides was observed<sup>21</sup> from uraninite not protected by organic matter in reactor core 2, which contains clays but virtually no organics. In contrast, sample 1 in reactor core 7, where uraninite is enclosed in organic matter, shows near-normal fission product ratios and insignificant migration of fissionogenic isotopes, especially neodymium (Table 2). On the basis of these measurements, clay minerals seem to be less effective than organic matter at preventing the release of uranium and fissionogenic isotopes.

Isotopic analyses by thermal ionization mass spectrometry



indicate varying uranium and lead mobilities. Uranium from bitumen in a vein at the edge of reactor core 9 shows no  $^{235}\text{U}/^{238}\text{U}$  depletion, whereas that from an unfractured sample shows  $^{235}\text{U}/^{238}\text{U}$  depletion, indicating influx of isotopically normal uranium through fractures after the reactor went critical. The isotopes  $^{238}\text{U}$  and  $^{235}\text{U}$  naturally decay to radiogenic, non-fissionogenic  $^{206}\text{Pb}$  and  $^{207}\text{Pb}$ , respectively, allowing determination of radiometric ages, which should be the same for coeval samples that were part of closed systems. Organic-rich samples 2, and 4–7 give dissimilar  $^{207}\text{Pb}/^{206}\text{Pb}$  ages (Table 2), indicating that the samples had been open systems with respect to uranium and lead migration. Migration of mobile intermediate decay products such as  $^{222}\text{Rn}$  and/or  $^{226}\text{Ra}$  (refs 22, 23) into samples 5 and 6, which have very low lead and uranium concentrations, would lead to excess  $^{206}\text{Pb}$  and might explain their low (and negative)  $^{207}\text{Pb}/^{206}\text{Pb}$  ages. Galena (sample 3) contains radiogenic lead with a  $^{207}\text{Pb}/^{206}\text{Pb}$  age of  $1,788 \pm 4$  Myr, indicating that this lead evolved in a uraniferous closed system until the late Phanerozoic. The three organic samples that contain sufficient uranium to plot on a concordia diagram (Fig. 1a) show considerable lead and/or uranium mobility. Data points from closed systems would lie on the concordia curve. Points above concordia are from samples that have lost uranium and/or gained radiogenic lead, points below are for samples that have gained uranium and/or lost radiogenic lead. Data from minerals suffering only one episode of lead-uranium mobility should lie along a line whose upper and lower concordia intersections give the ages of primary formation and mobilization, respectively. The scatter of data points around the best-fit line in Fig. 1a is outside analytical error, indicating that mobilization of lead and

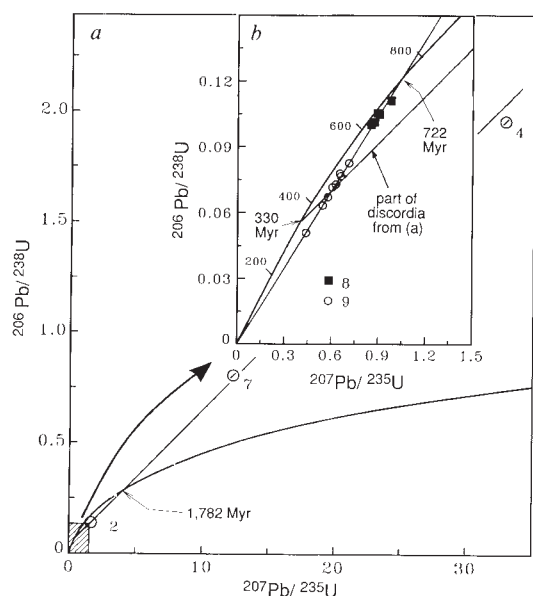


FIG. 1 a, Concordia plot showing U/Pb isotope analyses by thermal ionization mass spectrometry of three uraniferous organic samples from Oklo. The line is the calculated best-fit regression to the three points. The U/Pb ratios are very disturbed and the scatter in the points is well outside analytical error. Numbers 2, 4, 7, 8 and 9 refer to sample numbers in Tables 1 and 2. b, Concordia plot showing U/Pb isotope analyses by ion microprobe mass spectrometry of thirteen uraninite grains in organic matter in samples 8 and 9, located 200 m from reactor 9 and 50 m from a dolerite dyke intrusion. The best-fit line for discordia was calculated following the procedure of Ludwig<sup>27</sup>. The error is given at  $2\sigma$  (95% confidence level). Uranium and lead mobilization age ( $T_2$ ) is  $722 \pm 30$  Myr.  $T_1 = 1,850 \pm 15$  Myr defines the apparent time at which natural reactors 7–9 went critical. It was calculated as follows:  $(^{207}\text{Pb}/^{206}\text{Pb})_{\text{radiogenic}} = \text{present } (^{235}\text{U}/^{238}\text{U}) \times 10^{-2} \times [\exp(\lambda^{235}\text{U} T_1) - \exp(\lambda^{235}\text{U} T_2)] / [\exp(\lambda^{238}\text{U} T_1) - \exp(\lambda^{238}\text{U} T_2)]$ , where  $(^{207}\text{Pb}/^{206}\text{Pb})_{\text{radiogenic}}$  is the radiogenic lead in galena.  $T_1$  was calculated on the basis of  $^{207}\text{Pb}/^{206}\text{Pb} = 0.1405 \pm 0.0015$  ( $2\sigma$ ), which is the mean value of seven individual galena crystals analysed from reactors 7–9.

uranium occurred more than once. The lower concordia intersection of 330 Myr should represent an approximate average age of mobility. Although the points reveal substantial disturbance (50–80%), their scatter could be accounted for by a minimum of 300 Myr ( $330 \pm 150$  Myr) duration of mobility, suggesting containment long after criticality. The cause of this mobilization is unknown, because rocks of this age are absent in Gabon.

Individual uraninite grains were analysed by ion microprobe mass spectrometry. This method ensures that different mobilities of uranium and lead are recognized in uraninite crystals of different environmental histories. These grains were enclosed in graphitic organic matter in two samples located 200 m from reactor 9 and only 50 m from a major intrusive dolerite dyke of the dyke-swarm, which was dated<sup>24</sup> at  $750 \pm 150$  Myr. An upper concordia intersection of  $722 \pm 30$  Myr (Fig. 1b) was obtained<sup>25</sup>. Data points show low scatter along the discordia. An episode of uranium and lead mobilization, and uraninite and galena crystallization, therefore occurred at  $722 \pm 30$  Myr age and was caused by the intrusion of an adjacent ~15-m-wide dolerite dyke and the extensive dyke-swarm. Apparently the first large migration of U and Pb (and various fissionogenic isotopes) occurred at ~1,100 Myr after the reactors went critical at Oklo, because of the onset of a major, adjacent tectonic-igneous event. Some earlier migration, possibly caused by minor tectonic events, cannot be ruled out, but the limited scatter of data points 2, 7 and 4 (Fig. 1a) suggests that this was probably minor.

We conclude that immobilization of uraninite in solid graphitic organic matter enhanced the containment of  $^{235}\text{U}$  and a number of fissionogenic isotopes at the Oklo natural reactors. Therefore, we suggest that the same containment mechanism should be considered for the burial of anthropogenic nuclear waste products. It should be noted, however, that the very specific processes leading to immobilization at Oklo are not simply analogous to the enclosure of a radionuclide-containing inorganic solid in highly graphite bitumen. Nevertheless, other characteristics of solid bitumen, such as its hydrophobicity, inducible plasticity and nonflammability, should encourage us to explore this avenue for waste containment. □

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# Amyloid plaques, neurofibrillary tangles and neuronal loss in brains of transgenic mice overexpressing a C-terminal fragment of human amyloid precursor protein

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ALZHEIMER'S disease (AD) affects more than 30% of people over 80 years of age<sup>1,2</sup>. The aetiology and pathogenesis of this progressive dementia is poorly understood, but symptomatic disease is associated histopathologically with amyloid plaques, neurofibrillary tangles and neuronal loss primarily in the temporal lobe and neocortex of the brain. The core of the extracellular plaque is a derivative of the amyloid precursor protein (APP)<sup>3</sup>, referred to as  $\beta$ /A4 (refs 4–6), and contains the amino-acid residues 29–42 that are normally embedded in the membrane-spanning region of the precursor<sup>3</sup>. The cellular source of APP and the relationship of its deposition to the neuropathology of AD is unknown. To investigate the relationship between APP overexpression and amyloidogenesis, we have developed a vector to drive expression specifically in neurons of a C-terminal fragment of APP that contains the  $\beta$ /A4 region, and have used a transgenic mouse system<sup>7,8</sup> to insert and express this construct. We report here that overexpression of this APP transgene in neurons is sufficient to produce extracellular dense-core amyloid plaques, neurofibrillary tangles and neuronal degeneration similar to that in the AD brain.

Figure 1 shows the construction of the neuron-specific expression vector that we used in transgenic mice. The construct

consists of 3.6 kilobases (kb) of 5' flanking DNA of the human *Thy-1* gene, which is expressed in the central nervous system<sup>9,10</sup>, linked to the DNA fragment encoding the C terminus of APP. The APP fragment was chosen because it may be toxic to differentiated neurons in culture<sup>11</sup>. Four transgenic founder mice were produced, and two of three surviving lines that expressed the gene (lines 1 and 2) were used for this study. Expression was first observed on embryonic day 15, and increased to stable, high levels by two months of postnatal life in both lines. Figure 2a and b shows northern analysis of several adult transgenic and corresponding control mouse tissues from one expressing line. Expression is very high and is specific to brain. To determine the cell type that expressed the foreign gene, *in situ* hybridization was performed on brain using a labelled antisense cRNA probe specific for the 3' end of the human APP gene<sup>12</sup>. Results (Fig. 2c, d) show very high concentrations of APP mRNA in neurons throughout the brains of transgenic animals compared with non transgenic littermates. There was no hybridization with sense-strand RNA (data not shown).

Mice were examined for the effects of overexpression of this peptide in brains of animals of about 4 and 8 months old. At four months of age, transgenic mice from both lines had extracellular 'diffuse' amyloid deposits as large as 100  $\mu$ m in diameter and located primarily in the hippocampal formation, as visualized by anti- $\beta$ /A4-specific immunocytochemistry. Negative control brain tissue did not stain and preadsorbed serum elicited no immunoreactivity on transgenic tissues (data not shown). But no substantial amyloid deposits could be seen after Bielschowsky silver staining, nor were there any overt neuronal changes at this younger age.

At 8 months old, line 1, which showed higher transgene expression during fetal development, manifested severe pathology. Silver staining revealed dense-core plaques, neurofibrillary tangles and neuritic dystrophy (Fig. 3a, b). Plaques and tangles were limited to the hippocampal formation, amygdaloid complex and neocortex. In neocortex, the mean density of plaques and tangles as determined by Bielschowsky staining was  $36 \pm 15$  and  $51 \pm 17$  per 20- $\mu$ m section, respectively. This corresponds to 4 plaques per 10 mm<sup>2</sup>, and between 7 and 8 tangles per 10 mm<sup>2</sup>.

Immunocytochemical procedures revealed that these plaques and tangles strongly resembled those typically found in the brains of humans with AD: staining with Alz-50 and anti- $\beta$ /A4-specific antibodies demonstrated immunoreactivity of tangles and neuritic plaques. Dystrophic neurites were also visualized with Alz-50 (Fig. 3c). Specificity of anti- $\beta$ /A4 staining was

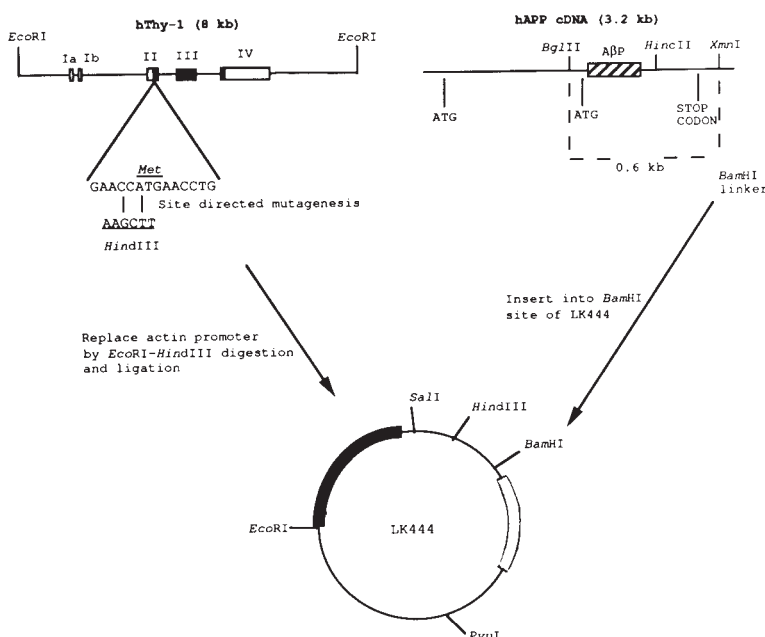
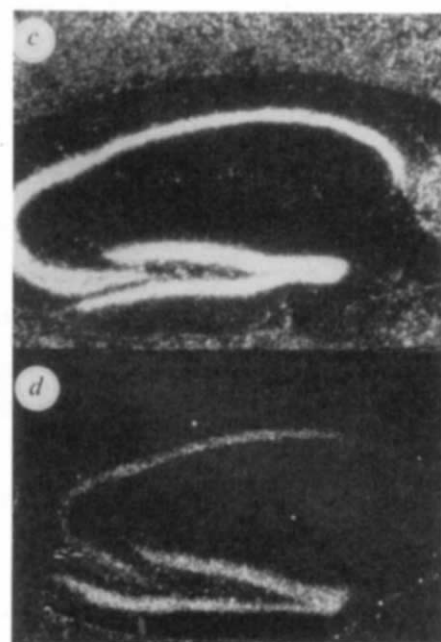
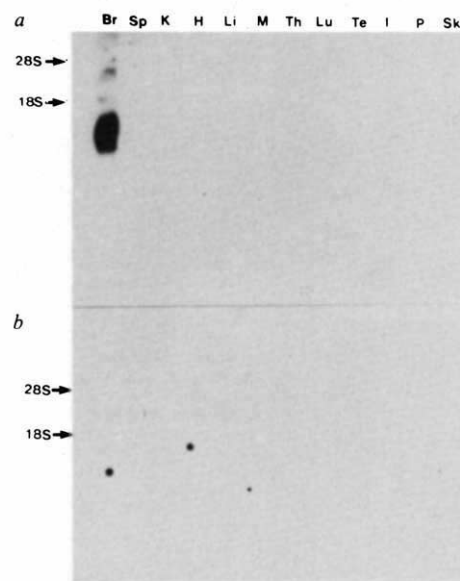


FIG. 1 Diagram of strategy for constructing the neuron-specific expression vector containing the C-terminal region of APP. To obtain the *Thy-1* element, an *EcoRI*-*Bgl*II 5' fragment that included the translation initiation codon was subcloned into an M13-derived vector (M13mp18; New England Biolabs) which was digested with *EcoRI* and *Bam*HI. Site-directed mutagenesis<sup>24</sup> was then used to alter two bases such that the ATG was destroyed and a *Hind*III site was created. The mutated fragment was then removed from the vector by *EcoRI* and *Hind*III digestion, and used to replace the  $\beta$ -actin promoter of the expression vector LK444 (ref. 25). The C-terminal region of APP was digested with *Xmn*I, equipped with *Bam*HI linkers, then digested with *Bam*HI and *Bgl*II and inserted into the *Bam*HI site of LK444. This fragment has an ATG sequence immediately upstream of the  $\beta$ /A4 region which was utilized as a translation initiation codon. The vector was digested at a unique *Pvu*I site for microinjection and partially with *Eco*RI to obtain 6.0-kb fragment. In the map of LK444 the shaded box represents the replaced  $\beta$ -actin promoter, and the open box indicates the SV40 intron and polyadenylation signal.



FIG. 2 Northern and *in situ* hybridization analyses of Thy-1-APP gene expression in transgenic mice. *a* and *b*, Northern analysis of a variety of tissues from a transgenic (*a*) and control (*b*) mouse. Lanes are labelled as follows: Br, brain; Sp, spleen; K, kidney; H, heart; Li, liver; M, skeletal muscle; Th, thymus; Lu, lung; Te, testis; I, intestine; P, pancreas; Sk, skin. *c* and *d*, Darkfield photomicrographs of *in situ* hybridization using a  $^{35}\text{S}$ -labelled RNA probe specific for a 3' portion of human APP messenger RNA corresponding to the restriction fragment used in the transgene. Sagittal sections were from 4-month-old animals: *c*, transgenic mouse, and *d*, non-transgenic littermate. A low level of cross hybridization to endogenous mouse APP mRNA is evident in *d*.

**METHODS.** All mice were obtained from the Jackson laboratory, Bar Harbor, Maine. Transgenic mice were produced by pronuclear microinjection<sup>7,26</sup> of B6D2F1  $\times$  B6D2F1 embryos, and founders were identified by screening DNA extracted from spleen by *Hind*III or *Bam*HI digestion and Southern<sup>27</sup> hybridization. Transgenic offspring were subsequently identified by tail biopsy and DNA extraction followed by Southern hybridization. Total RNA was purified from each tissue as described in ref. 28. Total RNA (15  $\mu\text{g}$ ) was run on formaldehyde/1.2% agarose gels and blotted onto nylon membranes. Filters were prehybridized with  $5 \times \text{SSPE}$  ( $1 \times \text{SSPE} = 0.18 \text{ M NaCl}$ , 10 mM sodium phosphate, 1 mM EDTA, pH 7.7),  $5 \times$  Denhardt's solution, 50% formamide, 20  $\mu\text{g ml}^{-1}$  salmon sperm DNA at 42 °C for 3 h and hybridized for 16 to  $3 \times 10^6$  c.p.m. of  $^{32}\text{P}$ -labelled *Hind*III-*Pvu*II fragment of the newly made plasmid. Blots were washed to a final



stringency of  $0.1 \times \text{SSPE}$  at 65 °C. Quality and quantity of RNA were confirmed by ethidium bromide staining and hybridization with a murine  $\beta$ -actin probe. For *in situ* hybridization, transgenic and control tissues were mounted on the same slide and hybridized simultaneously using 30 ng cRNA probe with a specific activity of  $1.1 \times 10^9$  c.p.m. The probe was generated from a 1.055-kb *Eco*RI fragment corresponding to bases 1,942–2,997 of APP-695 mRNA<sup>3</sup>, using a kit purchased from Promega Biotech (Riboprobe). *In situ* hybridization was performed as described<sup>12</sup>. Magnification, 200 $\times$ .

confirmed by peptide preadsorption (Fig. 3f). Thioflavin S-positive profiles were abundant in the hippocampus and cortex and were also visible in white matter (Fig. 4a) of 8-month-old animals of line 1. Cortex contained extracellular deposits and intracellular entities that were fluorescent on staining with thioflavin S (Fig. 4b).

Older animals of line 1 also showed extensive and widespread neuronal cell loss as compared with either 4-month-old transgenics or age-matched non-transgenic littermates. Neuronal cell counts showed a 40% reduction in the number of *Cornu Ammonis* pyramidal neurons of the hippocampal formation and of Purkinje cells of the cerebellum. This difference was highly

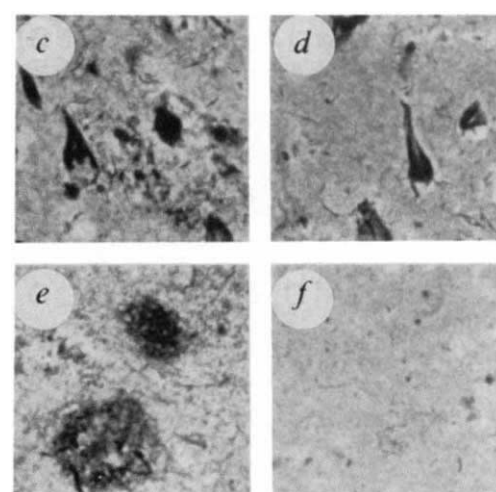
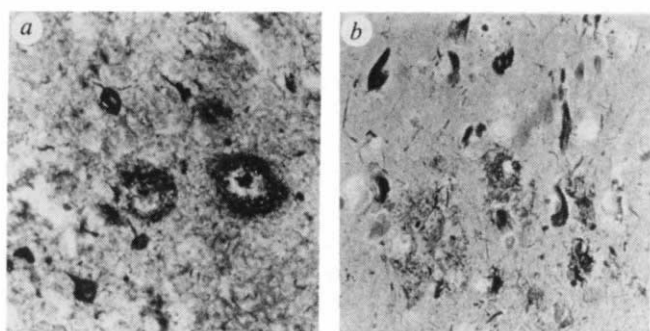
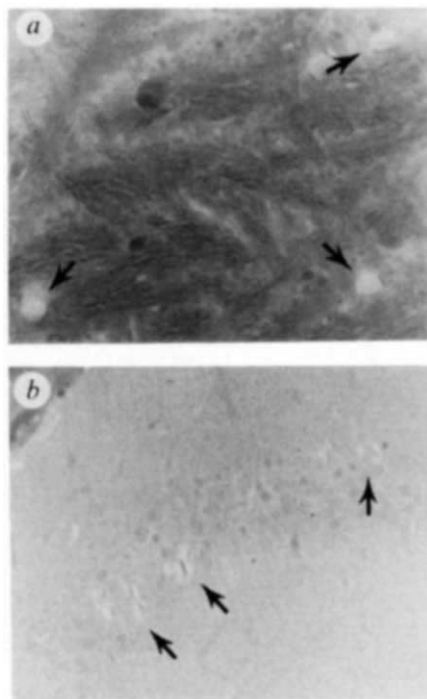


FIG. 3 Silver and immunocytochemical staining of amyloid plaques and neurofibrillary tangles in the neocortex of 8-month-old transgenic mice. *a* and *b*, Extracellular deposition of amyloid plaques and neurofibrillary tangles in the neocortex of an 8-month-old transgenic mouse from line 1 as revealed by silver staining. *a*, High-magnification view of dense-core plaques and tangle-bearing neurons ( $\times 350$ ); *b*, neuritic plaques and neurofibrillary tangles ( $\times 200$ ). *c*–*f*, Immunocytochemical staining of neurofibrillary tangles, neuritic plaques and dystrophic neurites. *c*, *d*, Alz-50; *e*, anti- $\beta$ /A4 immunohistochemical staining; *f*, anti- $\beta$ /A4 antibody staining of a section adjacent to that shown in *e* after preadsorption of the antibody with a synthetic peptide corresponding to residues 1–28 of the  $\beta$ /A4 protein ( $\times 350$ ).

**METHODS.** All tissues were sectioned at a thickness of 20  $\mu\text{m}$ . For silver staining, frozen tissue sections were processed using a modification of the Bielschowsky silver impregnation method<sup>29</sup>. For immunocytochemical procedures, a polyclonal anti- $\beta$ A4 antibody was prepared in rabbits using a

synthetic peptide corresponding to residues 1–28 of the  $\beta$ /A4 protein. The antibody was affinity-purified to the immunizing peptide. The same peptide was used as a preadsorption control by incubation with the primary antibody at a concentration of 100  $\mu\text{g ml}^{-1}$  at 37 °C for 1 h before immunocytochemical staining. The antibodies were used at the following dilutions: Alz-50, 1:500;  $\beta$ A4, 1:1,000. The immunoperoxidase procedure was performed as previously described using nickel sulphate intensification of the reaction product<sup>30</sup>.

FIG. 4 Thioflavin S staining of *a*, extracellular amyloid deposits (arrows) within the corpus callosum, and *b*, intracellular deposits within hippocampal pyramidal neurons (arrows) of an 8-month-old transgenic mouse from line 1. Frozen tissue sections (40- $\mu$ m thick) were stained with a 1% solution of thioflavin S and differentiated in acetic acid.



significant by Student's *T*-test ( $P \leq 0.0005$  for hippocampus,  $P \leq 0.005$  for cerebellum). Neuronal degeneration in the cerebellum was not associated with the presence of amyloid plaques or neurofibrillary tangles.

Previous evidence implicates excessive deposition of  $\beta/A4$  in the pathogenesis of AD. In Down's syndrome, where AD develops in all individuals over 30 years old, deposition of  $\beta/A4$  as 'diffuse plaques' without morphological alterations in neurites<sup>13,14</sup> precedes by 10–20 years the development of mature amyloid plaques<sup>15–18</sup>. But although this temporal sequence  $\beta/A4$  production and neuropathology is consistent with a causative relationship between the two events, the difficulty in isolating any of the processes that might contribute to AD has precluded identification of APP deposition as a cause of the disease. These transgenic mice demonstrate that a single disturbance, overproduction of the carboxy terminal portion of APP, can lead to all of the major neuropathological features observed in AD. Also unknown is the cellular origin of the  $\beta/A4$  in the amyloid plaques in AD<sup>12,19–21</sup>. The use of a promoter with neuronal specificity in these transgenic animals shows that production of  $\beta/A4$  protein from neurons alone can cause pathology similar to that seen in AD.

It is of interest to determine the precise portion of the APP C terminus that is contained in the plaques of these mice, as the transgene encodes a peptide that extends beyond the C-terminal limit of  $\beta/A4$ . We have stained plaques with an antibody specific for the 16 C-terminal amino-acid residues of APP and detected no staining. Although this result is consistent with the notion that the plaques contain authentic  $\beta/A4$ , we emphasize that the data are preliminary, and that only more research will rule out the possibility that the C-terminal region is absent rather than inaccessible to the antibody.

Others reported production of amyloid protein in brains of transgenic mice carrying various constructs of APP<sup>22,23</sup>, but none of these mice developed dense-core amyloid plaques, neurofibrillary tangles, thioflavin S-positive profiles or neuronal cell loss. Our Thy-1-APP transgenic mice have all of these neuropathological features of AD and so represent an important advance towards a rodent model for the disease. These animals could be used to study early manifestations of AD and to provide a test system for therapeutic intervention. □

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## A three-base-pair deletion in the peripherin-RDS gene in one form of retinitis pigmentosa

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THE group of retinopathies termed retinitis pigmentosa (RP) greatly contribute to visual dysfunction in man with a frequency of roughly 1 in 4,000 (refs 1, 2). We mapped the first autosomal dominant RP (adRP) gene to chromosome 3q (refs 3, 4), close to the gene encoding rhodopsin, a rod photoreceptor pigment protein. Subsequently, mutations in this gene have been implicated as responsible for some forms of adRP<sup>5–9</sup>. Another adRP gene has been mapped to chromosome 8p (ref. 10). A third adRP gene in a large Irish pedigree has been mapped to chromosome 6p (refs 11, 12), showing tight linkage with the gene for peripherin<sup>13,14</sup>, a photoreceptor cell-specific glycoprotein, which is thus a strong candidate for the defective gene. We have now identified a three-base-pair deletion which results in the loss of one of a pair of highly conserved cysteine residues in the predicted third transmembrane domain of peripherin. This deletion segregates with the disease phenotype but is not present in unaffected controls, and suggests that mutant peripherin gives rise to retinitis pigmentosa.

Human peripherin shows strong homology to the bovine outer segment membrane protein peripherin and to the normal protein product of the mouse retinal degeneration slow (*rds*) gene<sup>14–17</sup>. A mutation in the *rds* gene has been implicated as the underlying defect in the *rds* mouse<sup>16</sup>. These observations, together with our recently obtained close linkage between an adRP gene and the peripherin-RDS gene, stimulated us to screen 60 unrelated patients from adRP families for possible disease-causing



mutations in the peripherin-*RDS* gene. Using the complementary DNA sequence for the peripherin-*RDS* gene we designed a number of pairs of oligonucleotides to amplify the coding regions of the gene from these patients<sup>4</sup> using polymerase chain reactions (PCR). Single-strand conformation polymorphism electrophoresis was used to identify sequence alterations in amplified products<sup>18</sup>.

In this way we identified a DNA fragment, amplified from an adRP patient, with a greatly altered mobility. Direct sequencing of PCR products<sup>19</sup> revealed that this mobility shift is the result of a three-base-pair deletion at codon 118 or 119, which results in the loss of one of a pair of highly conserved cysteine residues in the predicted third transmembrane domain of the peripherin-*RDS* protein (Fig. 1). We designed allele-specific oligonucleotides corresponding to the normal and mutant sequences. Using these oligonucleotides we tested amplified DNA from members of the adRP family and found the mutation present in all affected individuals and absent from all unaffected individuals (Fig. 2). The deletion is also absent in 152 unrelated normal individuals and in the remaining 59 adRP families who were used in the study. This mutation is also seen on 12% nondenaturing polyacrylamide gels. Under these conditions homoduplexes for the normal and mutant alleles as well as two species of heteroduplex DNAs separate clearly (Fig. 2). In a number of patients we identified a second sequence alteration in the same DNA fragment, a C→T base change at position 558. But this change does not result in an amino-acid substitution.

The affected members of this family manifest a late-onset form of adRP, individuals noting difficulty with night vision typically in their early twenties, with later onset of symptoms caused by loss to peripheral visual fields. Electroretinographic assessment showed absent rod-isolated responses even in young affected individuals (early teens). Cone responses in young, affected members of the family were greatly delayed and in all cases reduced in amplitude. However, cone activity was still

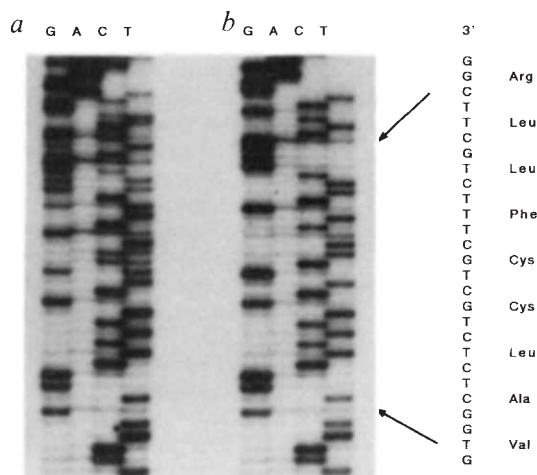


FIG. 1 Nucleotide and amino-acid sequences of codons 115-123 of the human peripherin-*RDS* gene from one patient with adRP (a) and an unrelated normal control individual (b). The adRP patient is heterozygous for a 3-bp deletion of either codon 118 or 119, in either case the sequence of the mutant allele will be identical and will result in the loss of a cysteine residue. The unaffected individual is homozygous for the normal sequence in this region.

**METHODS.** Using two oligonucleotide primers, 5'-TGCTATCCTGTGTCTTCAAC-3' and 5'-TCCCGAAACCGTGTGTC-3', we have PCR-amplified a 313-bp DNA fragment including the sequence coding for the third transmembrane domain of the peripherin gene, using 500 ng of leukocyte DNA from adRP patients and unaffected control individuals. The amplified products were directly sequenced using nested sequencing primers in the amplified fragment<sup>19</sup>. Sequencing primers: 5'-AAGTATGCCACATGGAAGCC-3', 5'-AGCGAGCCCGAAGCAGAAA-3', 5'-GTGTGTGTCGCGTAGTACT-3'.

detectable in affected individuals in their fifties. Visual field assessment indicated that patients tended to lose superior field before inferior and this was mirrored by greater pigment epithelial disturbance in the inferior retina. Funduscopy revealed the classic features associated with RP: disc pallor, retinal arteriolar attenuation and the presence of typical bone corpuscle pigment deposits.

This study is, to our knowledge, the first in which a naturally occurring animal disease model can be directly correlated to RP in man. Mice homozygous for the *rd*s mutation develop photoreceptors that fail to elaborate outer segments or outer-segment discs; photoreceptor degeneration occurs mostly within 12 months of birth<sup>20-22</sup>. Heterozygous *rd*s mice are also characterized by abnormal development of rod and cone photoreceptors followed by progressive degeneration, but they do form outer segments and photoreceptor degeneration is much slower than in homozygotes. The pathological changes observed in *rd*s mice show similarities to those present in RP patients<sup>23,24</sup>. Here we have provided evidence implicating a mutant peripherin-*RDS* protein as causative of one form of adRP. However, as both homozygous and heterozygous *rd*s mice show photoreceptor degeneration, it is possible that mutations in the human peripherin-*RDS* gene may also be involved in autosomal recessive RP.

Sequences are now available for the mouse, rat, bovine and human peripherin-*RDS* messenger RNAs<sup>14-17,25</sup>. Comparison

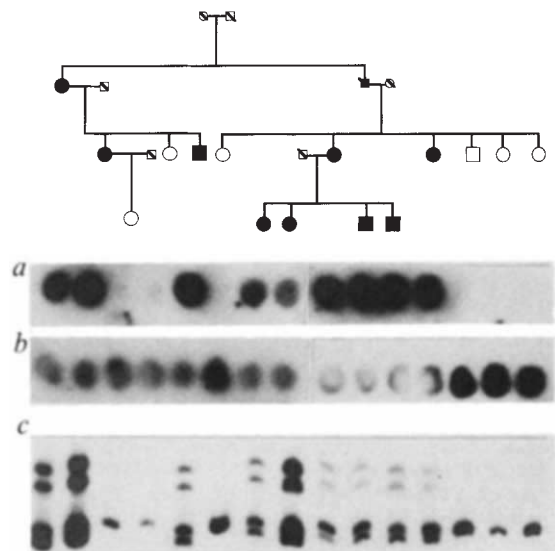


FIG. 2 Affected and unaffected individuals are indicated by filled and open symbols, respectively. The hybridization of both the mutant and the normal allele-specific oligonucleotides (ASOs) to amplified DNA fragments (including the region around the 3-bp deletion) from both affected and unaffected members are given below the pedigree. Only affected individuals hybridized to the mutant ASO (a). By contrast, all members of the pedigree hybridized to the normal ASO as expected (b). The mutant and normal alleles separate when run on 12% polyacrylamide gels (bands 3 and 4 in c). Furthermore, two species of heteroduplex DNA are visible (bands 1 and 2 in c). All affected individuals have both the normal and affected alleles and both species of heteroduplex DNA as expected. By contrast, the unaffected individuals have a single band of homoduplex DNA (band 3); that is, they are homozygous for the normal allele.

**METHODS.** DNA was amplified using the primers given in the legend to Fig. 1. Amplified products were dot-blotted onto nylon membranes and hybridized to  $\gamma$ -<sup>32</sup>P end-labelled mutant (5'-TGGCTCTCTGCTTTCTGCTT-3') and normal (5'-CTCTCTGCTGCTTTCTGCTT-3') ASOs<sup>27,28</sup>. Using a pair of oligonucleotides (5'-AAGTATGCCAGATGGAAGCC-3' and 5'-AGCGAGCCCGAAGCAGAAA-3'), a 110-bp fragment around the site of the 3-bp deletion was PCR-amplified incorporating [ $\alpha$ -<sup>32</sup>P]dCTP from both affected and unaffected members of the family. Amplified products were run on 12% polyacrylamide gels, which enabled the separation of the normal and mutant alleles (homoduplex DNA) and the two species of heteroduplex DNA.

of the predicted proteins demonstrates that the cysteine residues at codons 118 and 119 in the third transmembrane domain of the protein are highly conserved<sup>14,26</sup>, suggesting that the deletion of one of these cysteine residues may have a large effect on the protein structure and/or function, possibly resulting in the disruption of the membrane or alteration of protein stability.

Preliminary glimpses of a diverse molecular pathology in RP are now emerging. Rhodopsin and peripherin-RDS have both been implicated in adRP; both are photoreceptor-specific transmembrane proteins. Although the role of rhodopsin in light transduction is well established, the function of the peripherin-RDS protein is as yet unknown; however initial evidence indi-

cates that it may be involved in photoreceptor morphogenesis, possibly as an adhesion molecule for stabilization of outer segment discs<sup>23</sup>. Rhodopsin mutations have been found in the transmembrane, the cytoplasmic and the intradiscal regions of the protein in adRP patients, many of which seem to be specific to single families. Screening of large numbers of RP patients for further mutations will reveal if this is also the case with peripherin-RDS. The means by which these mutant proteins result in photoreceptor degeneration have still to be elucidated. But in the case of peripherin-RDS, the presence of a naturally occurring mouse model may be useful in this process. □

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## Mutations in the human retinal degeneration slow gene in autosomal dominant retinitis pigmentosa

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**THE murine retinal degeneration slow (*rds*) gene is a semidominant mutation with a phenotype having rod and cone photoreceptors that develop abnormally and then slowly degenerate<sup>1-3</sup>. The phenotype is a possible model for retinitis pigmentosa, one of the scores of hereditary human retinal degenerations, which is also characterized by photoreceptor degeneration. We report here three mutations of the human homologue of the *rds* gene (*RDS*) that cosegregate with autosomal dominant retinitis pigmentosa in separate families. Our results indicate that some cases of autosomal dominant retinitis pigmentosa are due to mutations at the *RDS* locus.**

Starting with a murine complementary DNA clone corresponding to the wild-type sequence<sup>4</sup>, we isolated the corresponding human cDNA sequence from a human retinal cDNA library<sup>5</sup>. The longest cDNA clone (pHRDS8) spanned the entire open reading frame. This probe detects at least three diallelic restriction-fragment length polymorphisms (RFLPs) (*Apa*I, *Dra*I, *Bgl*II) at the *RDS* locus, each with a minor allele frequency greater than 0.20, based on a set of 108 'control'

individuals without retinitis pigmentosa or a family history of the disease<sup>5</sup>. With the probe pHRDS8 and its cognate RFLPs we were able to search for defects in this gene in retinitis pigmentosa.

We limited our investigation to the autosomal forms of retinitis pigmentosa after mapping the *RDS* locus to human chromosome 6p (ref. 5). Patients found to have a mutant rhodopsin gene were excluded as such mutations are the cause of dominant retinitis pigmentosa in 20-30% of families<sup>6-11</sup>. Using Southern blotting techniques, we searched for gene deletions or rearrangements among 106 unrelated patients with dominant retinitis pigmentosa and 126 unrelated patients with recessive retinitis pigmentosa. No aberrant restriction fragments were observed, making it unlikely that deletions or gene rearrangements with breakpoints in the *RDS* locus are a common cause of dominant or recessive retinitis pigmentosa. We found an overrepresentation of the minor allele of the *Apa*I RFLP in the set of unrelated dominant patients (but not in the recessive patients). In fact, 52 out of 106 patients with dominant retinitis pigmentosa carried this allele compared with an expected 39.5 (chi-square, 6.31; d.f., 1; 0.02 > *P* > 0.01). This departure from Hardy-Weinberg equilibrium suggested that a proportion of the dominant patients might share a distant ancestor who had a pathogenic mutation at the *RDS* locus that was syntenic with the minor *Apa*I allele.

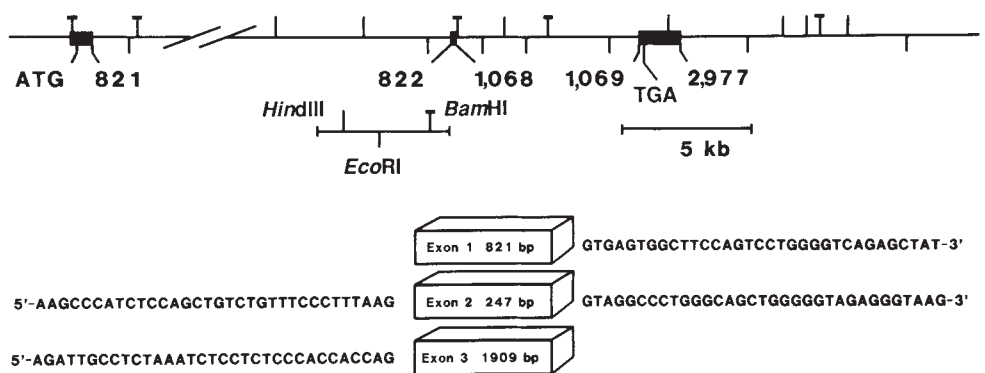
To explore this possibility it was necessary to determine whether some patients with dominant retinitis pigmentosa had mutations beyond the resolution of our Southern blotting techniques. An intron/exon map and a partial restriction map of the *RDS* locus were constructed (Fig. 1). We synthesized pairs of oligonucleotide primers in order to amplify segments of the coding sequence from genomic DNA using the polymerase chain reaction. The amplified DNA sequences were screened for mutations using single-strand conformation polymorphism analysis (SSCP)<sup>12</sup>. Three variant bands found during this investigation were of special interest. Each of these variant bands is due to an alteration in the DNA sequence that changes the encoded amino-acid sequence (Fig. 2). One variant is a three-base deletion (del) that precisely eliminates codon 219, which normally specifies proline. The second changes the specificity of codon 216 from proline to leucine, and the third changes the specificity

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FIG. 1 Map of the human *RDS* locus. The gene has two introns; the size of the first intron is unknown. Top, numbers beneath each exon denote the nucleotides at the 5' or 3' ends, based on the numbering scheme in ref. 5. Bottom, flanking intron sequences from which primers were derived.

**METHODS.** A human genomic library in the bacteriophage vector EMBL3 was probed with pHRDS8. Out of  $10.9 \times 10^6$  clones screened, 137 clones were positive. Six of these were plaque-purified, amplified and mapped. A consensus restriction map and the number and location of the exons were determined using standard methods. Restriction fragments containing exons were subcloned in plasmids and used as templates for obtaining intron sequences.



of codon 185 from leucine to proline. The first two variants, termed Pro 219→del and Pro 216→Leu, were each found in only one patient (AD206 and AD32, respectively) in an expanded set of 139 unrelated patients with dominant retinitis pigmentosa (all without a rhodopsin mutation); the third, Leu 185→Pro, was found in two unrelated patients (AD8 and AD145) from that set. None of these variants was found among an additional 52 unrelated patients with autosomal dominant retinitis pigmentosa who carry a rhodopsin mutation. None was present among 100 unrelated 'control' individuals without retinal degeneration. Finally, the relatives of three of these patients donated blood for analysis; in each family, the presence of the abnormality invariably correlated with the disease (Fig. 3). We conclude that each of these three variations represents a mutation that causes autosomal dominant retinitis pigmentosa.

Figure 4 displays the electroretinograms from an unaffected, control individual and patients with each of the three mutations. The left column shows the retinal response to a flash of dim blue light used to determine rod function. The middle column shows the response of both rods and cones to single flashes of bright white light. The right column provides the retinal response to flickering (30 Hz) white light; as rods cannot respond to this

flicker frequency, this is a measure of cone function. It can be seen that all patients with the mutations have abnormal electroretinograms; young patients have moderately reduced amplitudes and delayed response times. This is compatible with the notion that mutations in the *RDS* gene affect both rods and cones. Older patients with advanced disease showed profound reductions in both rod and cone amplitudes.

Despite the fact that the SSCP analysis was initiated in part because of the excess of an allele at the *ApaI* RFLP, we are as yet unable to find a mutation of the *RDS* locus, if any, that explains this finding. The *ApaI* RFLP is within the third exon (in the 3' untranslated region; data not shown), which we have so far only partially examined by the SSCP technique in the set of autosomal dominant patients. Additional mutations will probably be found when the entire coding sequence is analysed in every patient; one of these might explain the linkage disequilibrium with the *ApaI* RFLP.

The *RDS* gene is the second human locus, rhodopsin being the first<sup>6-11</sup>, at which mutations can cause autosomal dominant retinitis pigmentosa. The product of this gene is a membrane-associated glycoprotein confined to the photoreceptor outer segments<sup>13-15</sup>. On the basis of the phenotype of the *rd*s strain

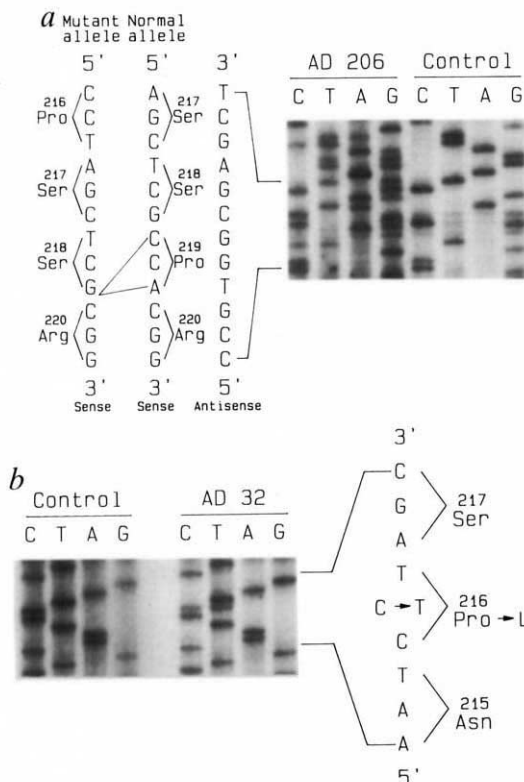


FIG. 2 Nucleotide sequence of codons altered in patients with autosomal dominant retinitis pigmentosa. *a*, Patient AD206 has a deletion of codon 219. *b*, Patient AD32 has a C-to-T transition in the second base of codon 216, changing the specificity of this codon from proline to leucine. *c*, Patient AD8 has a T-to-C transition in the second base of codon 185, changing the specificity of this codon from leucine to proline.

**METHODS.** The mutations in patients AD206 and AD32 were near the 5' end of exon 2, which was amplified by the polymerase chain reaction using the following primers: sense (derived from 3' end of the first intron), 5'-AAGCCCATCTCCAGCTGTCT-3'; antisense (derived from the middle of exon 2), 5'-TCGTAAGTGTAGTGCTGA-3'. The mutation in patient AD8 was near the 3' end of exon 1 which was amplified with the following primers: sense (derived from exon 1), 5'-TATGCCAGATGGAAGCCCTG-3'; antisense (derived from the 5' end of the first intron), 5'-TCTGACCCAGACTGGAAG-3'. The amplified DNA was sequenced directly<sup>17</sup>.

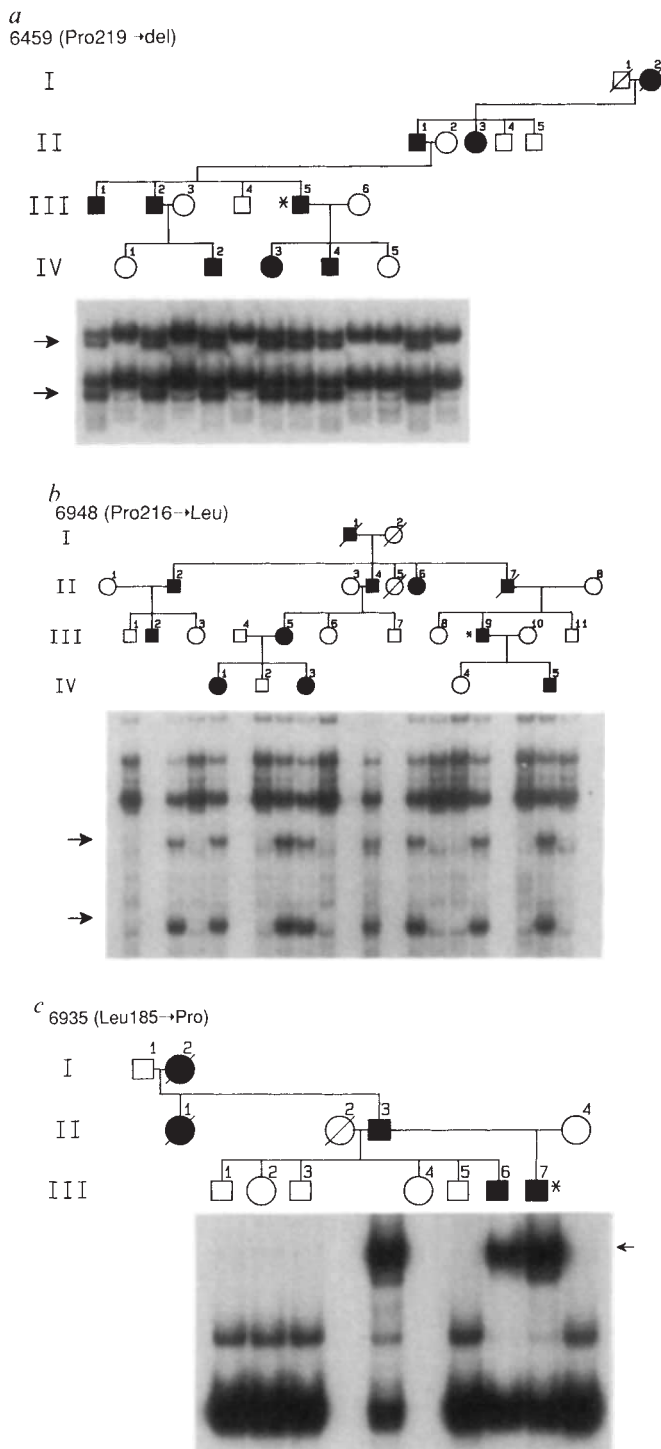


FIG. 3 Cosegregation of retinitis pigmentosa with the mutant bands detected by SSCP. Patients AD206, AD32 and AD8, with the mutations shown in Fig. 2, are members of families 6459, 6948 and 6935 (shown in *a*, *b* and *c*, respectively) and are indicated with asterisks. Affected members are designated by filled symbols. Beneath each schematic pedigree are the results of SSCP analysis. Arrows designate mutant bands. No blood sample was obtained from the individuals with blank lanes beneath their symbols. Patients IV-3 and IV-4 in pedigree 6459 and patient III-6 in pedigree 6935 have not been examined clinically.

**METHODS.** SSCP analysis was modified from ref. 12, using the primers described in the legend to Fig. 2, with the exception of the sense primer for family 6935, which was 5'-AGTACTACCGGACACAGAC-3'. DNA fragments were amplified by the polymerase chain reaction and digested with either *Dde*I (family 6948) or *Bst*NI (family 6935), or not at all (family 6459), before being denatured and separated by electrophoresis through a non-denaturing 6% polyacrylamide gel.

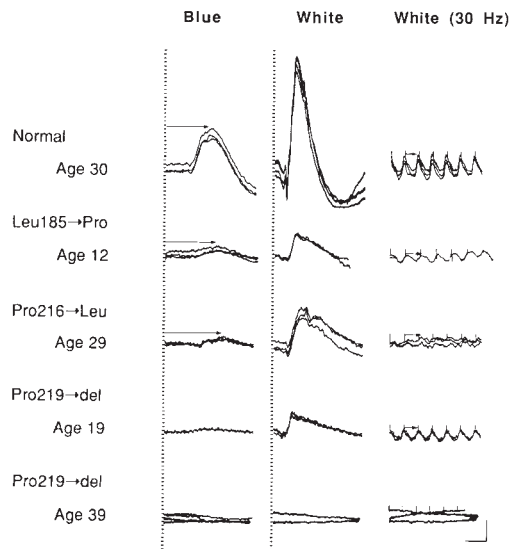


FIG. 4 Full-field electroretinograms from an unaffected individual (aged 30), patients with Leu 185 → Pro (aged 12), Pro 216 → Leu (aged 29), and Pro 219 → del (aged 19), each with an early stage of retinitis pigmentosa, and from a patient with Pro 219 del (aged 39) with an advanced stage of the disease. Illustrated are rod-isolated responses to flashes of dim blue light (left column), mixed cone-rod responses to flashes of white light (middle column), and cone-isolated responses to 30-Hz white flickering light (right column), using techniques described previously<sup>18,19</sup>. Stimulus onset is denoted by vertical dotted lines in the left and middle columns and by short vertical lines in the right column. Horizontal arrows in the left and right columns designate rod and cone response times respectively (that is, the time interval between a flash and the corresponding cornea-positive response peak). Under these test conditions, normal amplitudes are  $\geq 100 \mu\text{V}$  (left column),  $\geq 350 \mu\text{V}$  (middle column), and  $\geq 50 \mu\text{V}$  (right column); normal rod response time is  $\leq 108 \text{ ms}$  and normal cone response time is  $\leq 32 \text{ ms}$ . Calibration symbol (lower right) designates 50 ms horizontally and 100  $\mu\text{V}$  vertically.

of mice and its preliminary characterization, the protein probably helps maintain the structure of outer segment discs. The messenger RNA sequence has been determined in mouse<sup>4</sup>, human<sup>5</sup>, cow<sup>14</sup> and rat<sup>16</sup>. The three amino acids affected by the mutations described here are probably located in the second intradiscal loop<sup>13-15</sup>, a domain that is very highly conserved among these four mammalian species. In particular, the three residues involved in these mutations are invariant. The region also contains the only glycosylation site conserved in all four species. Substitutions between proline and leucine, or the loss of a proline residue, are non-conservative changes that can be expected to have major effects on the secondary structure of the protein.

The *rd*s allele in mice is most probably a *null* mutation, because it is due to the insertion in the reading frame of  $\sim 10$  kilobases (kb) of extraneous murine DNA, resulting in the truncation of the carboxy-terminal third of the protein<sup>4</sup>. We cannot exclude the possibility that the three mutations described here also represent *null* mutations that abolish some important function of the encoded protein. It also remains to be determined whether recessive mutations of this gene exist in humans and whether they cause photoreceptor cell degeneration. We plan to use the SSCP technique to search for mutations of the *RDS* locus in patients with recessively inherited photoreceptor cell degenerations, including recessive retinitis pigmentosa. □

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## Homology of a candidate spermatogenic gene from the mouse Y chromosome to the ubiquitin-activating enzyme E1

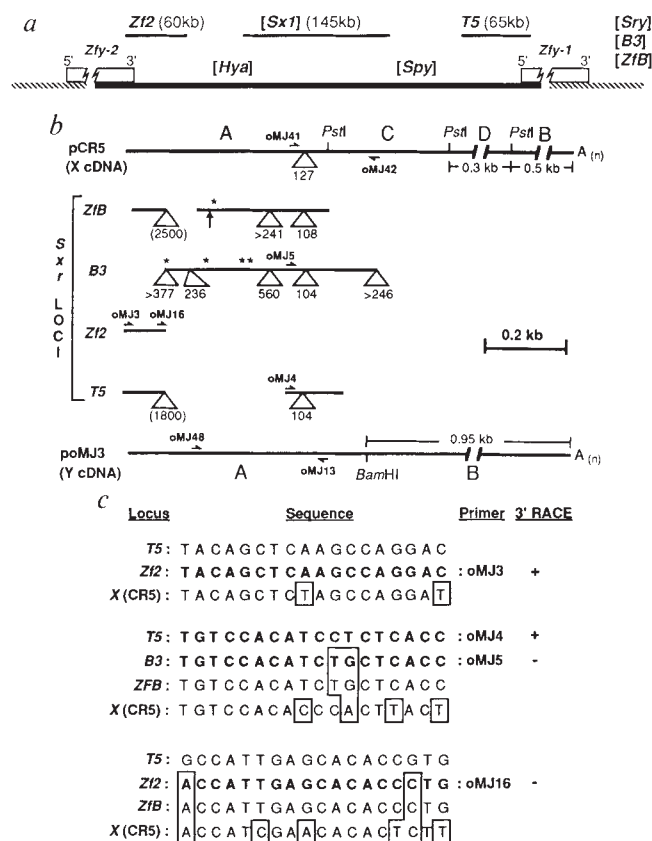
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**THE *Sxr* (sex-reversed) region, a fragment of the Y chromosome short arm, can cause chromosomally female XX*Sxr* or X*Sxr*O mice to develop as sterile males<sup>1-3</sup>. The original *Sxr* region, termed *Sxr*<sup>a</sup>, encodes: *Tdy*, the primary sex-determining gene; *Hya*, the controlling or structural locus for the minor transplantation antigen H-Y (ref. 4); gene(s) controlling the expression of the serologically detected male antigen (SDMA)<sup>5</sup>; *Spy*, a gene(s) required for the survival and proliferation of A spermatogonia during spermatogenesis<sup>6,7</sup>; *Zfy-1/Zfy-2*, zinc-finger-containing genes of unknown function<sup>8</sup>; and *Sry*, which is probably identical to *Tdy* (ref. 9). A deletion variant<sup>10</sup> of *Sxr*<sup>a</sup>, termed *Sxr*<sup>b</sup>, which lacks *Hya*, SDMA expression, *Spy* and some *Zfy-2* sequences, makes positional cloning of these genes possible. We report here the isolation of a new testis-specific gene, *Sby*, mapping to the DNA deleted from the *Sxr*<sup>b</sup> region (the  $\Delta$ *Sxr*<sup>b</sup> interval). *Sby* has extensive homology to the X-linked human ubiquitin-activating enzyme E1 (ref. 11). The critical role of this enzyme in nuclear DNA replication<sup>12</sup> together with the testis-specific expression of *Sby* suggests *Sby* as a candidate for the spermatogenic gene *Spy*.**



**FIG. 1** The isolation of  $\Delta$ *Sxr*<sup>b</sup> DNA and the identification of exonic sequences on the Y chromosome. **a**, The  $\Delta$ *Sxr*<sup>b</sup> DNA is represented by the thick black line and the *Sxr* DNA remaining in the *Sxr*<sup>b</sup> region by the broken line. The breakpoints of the deletion are shown by the gaps in the *Zfy* genes. The position of the breakpoints together with the indicated 3'-to-5' orientation of the *Zfy* genes and their order with respect to *Hya*, *Spy* and *Sry* are as determined previously<sup>19</sup>. The positions of the *T5*, *Zf2* and *Sx1* contigs are shown at the top with the size of each in parentheses<sup>13</sup>. Loci in square brackets have not been definitively mapped in relation to *Zfy* genes. *Hya*, *Spy* and *Sx1* must map between the *Zfy* genes and *Sry*. *B3* and *ZfB* must map outside the *Zfy* genes. **b**, Alignment of the *Sxr* genomic sequences with the pCR5 cDNA. The positions of the introns are indicated by triangles and their sizes (bp) where known are shown at the base of the triangle.

Intron sizes in parentheses were calculated from PCR data (the difference in size between the genomic and cDNA products for a given pair of primers) and the others were obtained directly from the genomic sequence. The single X intron sequenced had no homology with the corresponding Y introns. Stop codons in the same frame as the CR5 open frame are marked by asterisks and the vertical arrow indicates a frameshift mutation indicating that the *B3* and *ZfB* homologues are pseudogenes. The *B3* sequence was derived from pY8 and is presented in Tucker *et al.*<sup>20</sup>. The locations of primers are represented by the horizontal arrows. The subsequently cloned Y cDNA pMJ3 is shown at the bottom of the lineup. The origins of pCR5 subclones A-D and pMJ3 subclones A and B are indicated as are the positions of the primers used in later expression studies (oMJ13, 48, 41 and 42). **c**, Primer sequences used in 3' RACE experiments aligned with the corresponding sequences from the other loci. In each block all sequences are compared with the *T5* DNA and differences are boxed. The locus of origin of each sequence is indicated. Sequences from which primers were synthesized have the primer's name to their right and are in bold. The ability of a primer to generate a specific 3' RACE product is indicated by + or -. **METHODS.** The X cDNA CR5 was isolated from an adult testis library made from 129/sv/Pas testis poly(A) RNA inserted into the *EcoRI* site of  $\lambda$ NM1149 and subcloned into pBluescript II (Stratagene). The phage library was constructed by conventional methods and screened with pY8. To isolate potential exons, cloned DNA from the *ZfB*, *Zf2* and *T5* loci was restricted, Southern blotted and hybridized with pCR5.A. Positive fragments were excised from gels purified by freeze-fracture in liquid nitrogen with an equal volume of equilibrated phenol and subcloned into pBluescript II. Smaller fragments of ~0.3 kb were similarly subcloned from these recombinants. These latter subclones, together with pY8 (which was assigned to the *B3* locus<sup>13</sup>) and pCR5, were sequenced directly from double-stranded plasmid by the dideoxy chain termination method<sup>21</sup> using Sequenase version 2.0 (USB) and a combination of nested deletion sets and oligonucleotide primers. Sequence comparison was performed using the University of Wisconsin genetics computer group sequence analysis software package<sup>22</sup>. The 3' RACE<sup>23</sup> was done essentially as in ref. 24 using total RNA from C57BL/6 adult testis, prepared according to ref. 25. Different conditions were used for the PCR reaction which was run in 10 mM Tris-HCl pH 8.4, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.001% gelatin, 0.2 mM each dNTP and 25 pmol each primer. Only the annealing temperature was varied in the cycling parameters. Specific CR5/C/D homologous products were observed for oMJ3 and oMJ4 at 62 °C but not for oMJ5 or oMJ16 at temperatures above 56 °C. No products were amplified with any primers when the reverse transcriptase was omitted from the first strand synthesis.

With the expectation that an *Sxr* gene would be conserved on other mammalian Y chromosomes, 270 kilobases (kb) of  $\Delta Sxr^b$  DNA, previously isolated by chromosomal walking<sup>13</sup>, was used to screen 'zoo' blots of DNA from a male and female human, horse, pig and rabbit. A 5-kb *Eco*RI fragment, termed icZf.1.8.F, mapping about 20 kb 3' to the zinc-finger exon of *Zfy-2*, detected male-specific fragments in all species tested, with the exception of human (data not shown). Analysis of the region into which icZf.1.8.F maps (*Zf2*) (Fig. 1a) shows it to be complex, containing dispersed sequences that are present in at least five copies in *Sxr*. Contig sequences have been established from four of these related loci<sup>13</sup>. A homologue of the multi-copy *Sxr* probe pY8 (ref. 2), previously shown to detect a 3.5-kb transcript in adult testis<sup>14</sup>, also maps into the *Zf2* contig. Screening an adult testis complementary DNA library with pY8 resulted in the isolation of pCR5, a 1.6-kb partial cDNA. This

cDNA was mapped, using somatic cell hybrids, to the proximal portion of the mouse X chromosome (C. E. B. and P. Avner, unpublished results). The hybridization pattern of pCR5 to the zoo blots also revealed male-specific fragments and thus pCR5 represents the X homologue of a new Y gene and could be used to isolate exons rapidly from the cloned *Sxr* DNA. The contig DNA was, therefore, screened with pCR5 and homologous fragments were isolated and sequenced from two  $\Delta Sxr^b$  loci, *Zf2* and *T5* and two *Sxr^b* loci, *B3* and *ZfB*. A comparison of these genomic Y sequences with the pCR5 cDNA sequence revealed regions of homology adjacent to regions lacking homology, probably representing exons and introns, respectively (Fig. 1b).

The sequence information was used to design primers from each locus for the isolation of specific transcripts using the 3' RACE technique (3' rapid amplification of cDNA ends). These

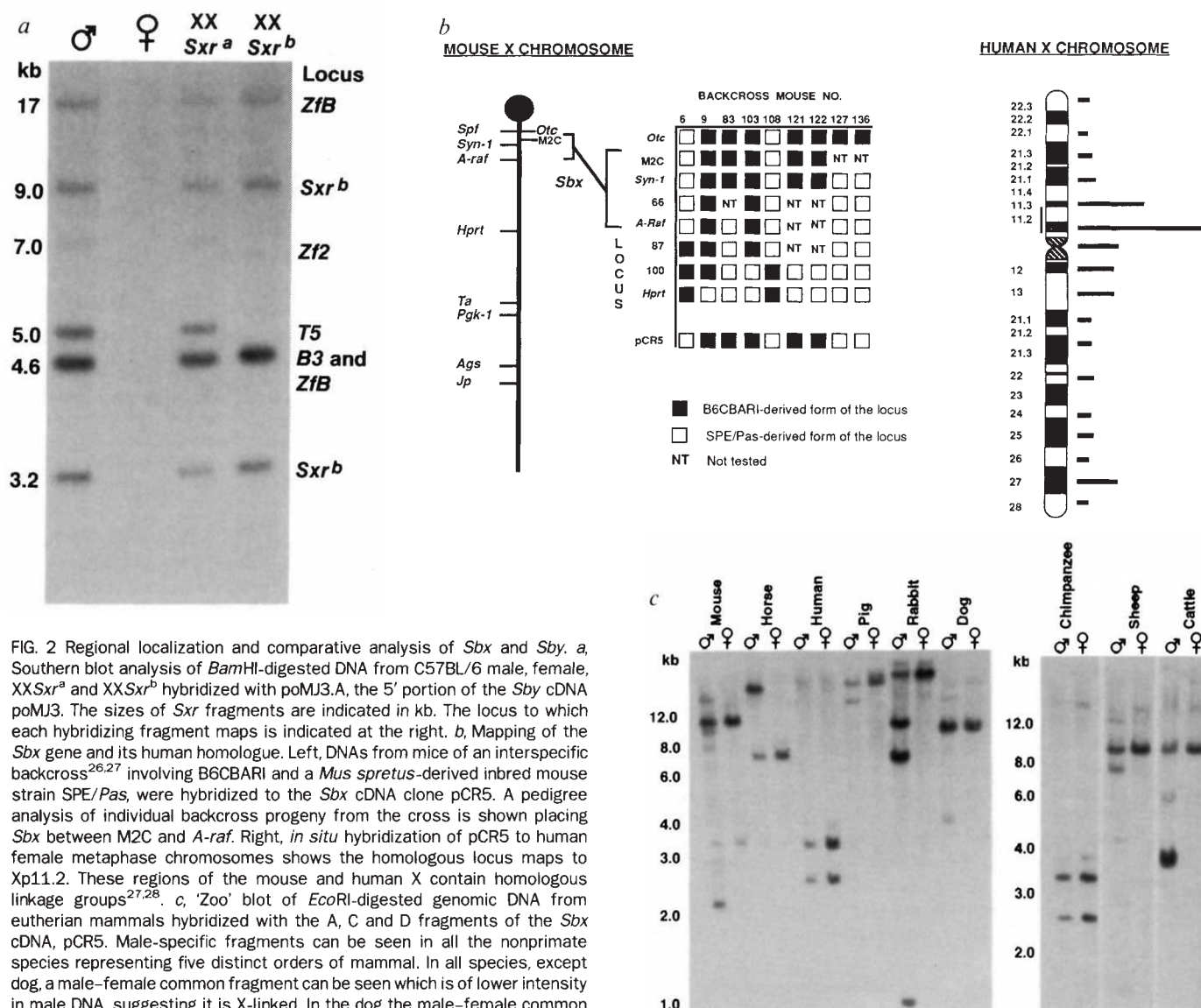


FIG. 2 Regional localization and comparative analysis of *Sbx* and *Sby*. **a**, Southern blot analysis of *Bam*HI-digested DNA from C57BL/6 male, female, XX*Sxr*<sup>a</sup> and XX*Sxr*<sup>b</sup> hybridized with pMJ3.A, the 5' portion of the *Sby* cDNA pMJ3. The sizes of *Sxr* fragments are indicated in kb. The locus to which each hybridizing fragment maps is indicated at the right. **b**, Mapping of the *Sbx* gene and its human homologue. Left, DNAs from mice of an interspecific backcross<sup>26,27</sup> involving B6CBARI and a *Mus spretus*-derived inbred mouse strain SPE/Pas, were hybridized to the *Sbx* cDNA clone pCR5. A pedigree analysis of individual backcross progeny from the cross is shown placing *Sbx* between *M2C* and *A-raf*. Right, *in situ* hybridization of pCR5 to human female metaphase chromosomes shows the homologous locus maps to Xp11.2. These regions of the mouse and human X contain homologous linkage groups<sup>27,28</sup>. **c**, 'Zoo' blot of *Eco*RI-digested genomic DNA from eutherian mammals hybridized with the A, C and D fragments of the *Sbx* cDNA, pCR5. Male-specific fragments can be seen in all the nonprimate species representing five distinct orders of mammal. In all species, except dog, a male-female common fragment can be seen which is of lower intensity in male DNA, suggesting it is X-linked. In the dog the male-female common fragment is of greater intensity in the male than the female DNA which suggests the presence of an additional male-specific fragment of the same size.

**METHODS.** **a**, Restricted DNA (8  $\mu$ g) was run in each lane of a 0.8% agarose gel in 1  $\times$  TAE (0.04 M Tris-acetate, 0.001 M EDTA). The electrophoresed DNA was transferred to Hybond-N membrane (Amersham) and fixed at 80 °C for 2 h. The membrane was prehybridized and hybridized in 0.5 M NaPO<sub>4</sub>, pH 7.2; 7% SDS<sup>29</sup> at 68 °C for 36 h. After hybridization, the membranes were washed 3  $\times$  20 min in 1  $\times$  SSC, 0.2% SDS at 65 °C. The probe was labelled with <sup>32</sup>P using random hexamer primers<sup>30</sup>. **b**, For the backcross mapping of *Sbx*, DNAs restricted with *Taq*I to reveal a B6CBARI/SPE/Pas

RFLP were hybridized with pCR5. The *M. spretus*- or B6CBARI-derived allele was identified and compared with previously typed markers to arrive at a map position. *In situ* hybridization, using cells collected according to the bromodeoxyuridine method<sup>31</sup> was done using <sup>3</sup>H-labelled pCR5 essentially as previously described<sup>32</sup>. One hundred metaphase spreads were scored and a total of 412 grains counted of which 84 (20.4%) were on the X chromosome with a peak at Xp11.2. No significant hybridization was seen on any of the autosomes (the Y was not available for scoring as female cells were used). **c**, Southern blot performed as in **a** except hybridization was done at 58 °C and the filters washed at 2  $\times$  SSC, 0.2% SDS at 55 °C.



primers are shown in Fig. 1c compared with the corresponding sequences from the other X and Y loci where known. Only primers oMJ3 and oMJ4, which are identical to sequences from the *T5* locus, amplified 3' RACE products, of 1.6 kb and 1.2 kb, respectively. Primers identical to sequences from the *ZfB* and *Zf2* loci (oMJ16) or the *B3* locus (oMJ5), but not the *T5* locus, failed to amplify a specific product. The cDNA for oMJ3 and oMJ4 products were cloned and named poMJ3 and poMJ4. Southern analysis with a 600-base pair (bp) fragment from poMJ3, poMJ3.A, revealed an almost exclusively *Sxr* hybridization pattern (Fig. 2a) and the bands were further assigned to individual loci by hybridizing poMJ3.A to the contig DNA from the *B3*, *ZfB*, *T5* and *Zf2* loci (data not shown). Thus, we have identified and cloned a transcript which is identical to sequences in the *T5* locus and conclude that this putative gene maps to the  $\Delta Sxr^b$  region. We have termed this gene *Sby* (for  $\Delta Sxr^b$  Y homologue) and the X homologue represented by cDNA pCR5, *Sbx*. Two nondeleted, *Sxr*<sup>b</sup> fragments of 3.2 and 9.0 kb (see Fig. 2a) do not map to the *B3*, *ZfB*, *T5* or *Zf2* contigs. Thus the *T5* locus may not be the only *Sxr* locus from which an *Sby* transcript

is expressed. *Sbx* was mapped in the mouse to the M2C/*A-raf* subcentromeric interval and in the human to the homologous Xp11.2 interval (Fig. 2b).

The *Sbx* cDNA detects homologues on the sex chromosomes of other mammals more effectively than the *Sby* cDNA and when used to analyse DNA from a male and a female mouse, human, horse, pig, rabbit, dog, chimpanzee, sheep and cow it can clearly be seen that CR5 hybridizes to male-specific restriction fragments in all but the primate DNA (Fig. 2c). Male-female common fragments can also be detected in all species tested including primates. These fragments appear fainter in males than females consistent with X-linkage. We are now attempting to screen for human Y homologues at reduced stringencies but it remains a strong possibility that, in primates, the homologous X gene compensates for the lack of the Y-linked homologue.

The *Sby* cDNA, poMJ3, was sequenced and found to be 1,511 bp long with an open reading frame (ORF) across nucleotides 1–1,326. The sequence was 100% identical to the putative exons previously sequenced from the *T5* genomic DNA but

|             |                                                                                     |
|-------------|-------------------------------------------------------------------------------------|
| 1           |                                                                                     |
| Hum-E1/A1S9 | TESYSSSQDPPEKSIPICTLKNEFPNAIEHTLQWARDEFEGFLKQPAENVNQQYLTDPKPFVERTLRLAG.TQPLEVLEAVQR |
| Mus-Sbx     | TESYSSSQDPPEKSIPICTLKNEFPNAIEHTLQWARDEFEGFLKQPAENVNQQYLTDSKPFVERTLRLAG.TQPLEVLEAVQR |
| Mus-Sby     | YSSSQDPPEKSIPICTLKNEFPNAIEHTVQWARDEFEGFLKQSAENVNQQYLTDPKFMERTLQLAG.TQPLEVLEAIHC     |
| Yeast-E1    | TESYSSSRDPPEKSIPLCTLRSEPNKIDHTIAWAKSLFQGYFTDAENVNMYLTQPNFVEQTLKQSG..DVKGVLSEISD     |
| Wheat-E1    | TENYGASRDPEKQAPMCTVHSEPHNIDHCLTWARSEFEGGLEKTPTEVNAFLSNPTTYISAARTAGDAQARDQLERVIE     |
| 81          |                                                                                     |
| Hum-E1/A1S9 | SLVLQRPQTWADCVTWACHHHWHTQYSNNIRQLLHNFPPDQLTSSGAPFWSGPKRCPHPLTFDVENNPLHLDDYVMAAANLFA |
| Mus-Sbx     | SLVLQRPQTWADCVTWACHHHWHTQYCNIRQLLHNFPPDQLTSSGAPFWSGPKRCPHPLTFDVENNPLHLDDYVMAAANLFA  |
| Mus-Sby     | SLVLQRPQTWADCVTWAYQHWHHTQYSHNIQQLLHNFPPAQLTSSGALFWSGPKRCPHPLTFDINNPLHLDDYVMAAANLFA  |
| Yeast-E1    | SLS.SKPHNFEDCIKWARLEFEKKENHDIKQLLEFNPKDAKTSNGEPFWSGAKRAPTPLEFDIYNNDHFHFVVAGANLRA    |
| Wheat-E1    | CLDRDKCETFDQSITWARLKFEFYFSNRVKQLTFTFPEDSMTSSGAPFWSSAPKRFPRPVEFSSSDQSQSLFIAAAAILRA   |
| 161         |                                                                                     |
| Hum-E1/A1S9 | QTYGLTGSQDRAAVATF.....LQSVQVPEFTPKSGVKIHSVSDQELQSANAS.VDDSR.LEELKATLPSDPKLP..GFKM   |
| Mus-Sbx     | QTYGLTGSQDRAAVASL.....LQSVQVPEFTPKSGVKIHSVSDQELQSANAS.VDDSR.LEELKATLPSDPKLP..GFKM   |
| Mus-Sby     | QTYGLGGSQDCAVAKL.....LQSLPVPKFAKPSGIRIHVSEQELQSTSATTIDDSH.LEELKTALPTPDKLL..GFKM     |
| Yeast-E1    | YNYGIKSDSSNSKPNVDEYKSVIDHMIPEFTPNANLKIQVNDPDPNANAANGSDE.IDQLVSSLPDPSTLA..GFKL       |
| Wheat-E1    | ETFGIPIPEWAKTPNKLAAEA.VDKVIVPDFQPKQGVKIVTHEKATSLSSAS.VDDAAVIEELIAKLEEVSKTLPSPGFHM   |
| 241         |                                                                                     |
| Hum-E1/A1S9 | YPIDFEKDDDSNFHMDFIVAASNLAENYDIPSAHRHKSCLIAGKIIIPAIATTTAAVVGVLVCELELYKVVQGHRLDSYKN   |
| Mus-Sbx     | YPIDFEKDDDSNFHMDFIVAASNLAENYDISPADRHKSCLIAGKIIIPAIATTTAAVVGVLVCELELYKVVQGHRLDSYKN   |
| Mus-Sby     | YPIDFEKDDDSNFHMDFIVAASNLAENYGISPADRHKSCLIAGKIIIPAIATTTAAVVGVLVCELELYKVVQGHRLDSYKN   |
| Yeast-E1    | EPVDFEKDDDTNHHIEFITACSNCAQNYFIETADRQKTKFIAGRIIPAIATTTSLVTGLVNLLEYKLVKIDNKTDIEQYKN   |
| Wheat-E1    | NPIQFEKDDDTNFHMDVIAGFANMRARNYSIPEVDKCLKAKFIAGRIIPAIATSTAMATGLVCELELYKALAGGHKVEDYRN  |
| 321         |                                                                                     |
| Hum-E1/A1S9 | GFLNLALPFFGFSEPLAAPRHQYYNQEW.TLWDRFEVQGLQPNGEEMTLKQFLDYFKTEHKLEITMLSQGVSMLYSFFMP    |
| Mus-Sbx     | GFLNLALPFFGFSEPLAAPRHQYYNQEW.TLWDRFEVQGVQPNGEEMTLKQFLDYFKTEHKLEITMLSQGVSMLYSFFMP    |
| Mus-Sby     | SFINLALPLFSFSAPLAPECHQFYDQEW.TLWDRFDVQGLQPSGEEMTLKQFLDYFKTEHKLEVIMLSQGVSMLYSVFMP    |
| Yeast-E1    | GFVNALPFFGFSEPIASPKGEYNNKKYDKIWDREFDIK.....DIKLSDLIEHFEKDEGLEITMLSYGVSLLYASFFP      |
| Wheat-E1    | TFANLAIPLFISIAEPVPPKTIKHQELSW.TVWDRWTVTG.....NITIRELLEWLK.EKGLNAYSISCGTSLLYNSMFP    |
| 401         |                                                                                     |
| Hum-E1/A1S9 | AAKLKERLDQPMTEIVSRVSKRKLGRHVRALVLELCCNDESGEDVEVPYVRYTIR*                            |
| Mus-Sbx     | AAKLKERLDQPMTEIVSRVSKRKLGRHVRHWCLSCAATMKAARTSRSLMSDIPFADLRAPP*                      |
| Mus-Sby     | ASKLKERLDQPMTEIVSCVSKQKLGHVHVKSLVFELCCNSDSGGDIEVPYVRYTIR*                           |
| Yeast-E1    | PKKLKERLNLPIQLVVKLVTKKIDIPAHVSTMLEICADDKEGEDVEVPFITIH*                              |
| Wheat-E1    | ..RHKERLDRKVVDAVEVAKMEVPSYRRHLDDVVDVACEDDDNDVDIPLVSVYFR*                            |

FIG. 3 Comparison of the partial deduced amino-acid sequence (single-letter code) of *Sby* to those of *Sbx*, the A1S9 cDNA<sup>15</sup> and the ubiquitin-activating enzymes (E1) from human<sup>11</sup>, wheat<sup>16</sup> and yeast<sup>17</sup>. The human E1 sequence includes the corrections published<sup>33</sup>. As A1S9 and the corrected human E1 sequences encode identical peptides in this region, they are presented together as hum-E1/A1S9. Positions of identity with *Sby* and conservative substitutions are shaded. Underlined, *Sbx* differs from *Sby* through the loss of a single base from the CR5 cDNA. This is an accurate representation of the sequence of the CR5 clone but may represent a rare cloning artefact.

This will be clarified by the sequencing of further cDNA clones. Dots represent gaps introduced to maintain alignment. The nucleotide sequences from which the *Sby* and *Sbx* amino-acid sequences were deduced have been deposited in the Genbank database accession numbers in the EMBL data library: *Sbx*, X62580; *Sby*, X62581.

METHODS. The *Sby* cDNA poMJ3 was subcloned into the *Sma*I and *Sal*I sites of pBluescript II and sequenced as described in the legend to Fig. 1. Comparisons at the nucleotide and amino-acid level were done using the University of Wisconsin GCG sequence analysis software package<sup>22</sup>.

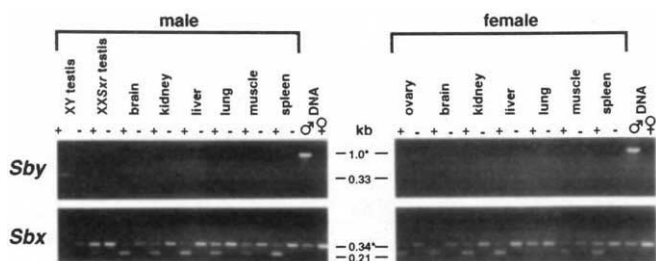


FIG. 4 Expression patterns of *Sby* and *Sbx* in adult tissues. Expression was studied using RT-PCR from total RNA from a variety of male and female tissues and XXSxr<sup>a</sup> testis. +, -, Presence or absence of reverse transcriptase during the first strand cDNA synthesis respectively. The sizes of fragments amplified are indicated in (kb) and the products amplified from genomic DNA are indicated with an asterisk. The RT-independent fragment amplified from the RNA samples by oMJ41/42 arises from DNA contamination. The lack of an RT-dependent product with the *Sbx* primers in female lung is probably due to degradation of the RNA in this sample as the same primers amplify a product in male lung.

**METHODS.** Total RNA was prepared from tissues following the protocol in ref. 25. The RNA was not DNase treated. RT-PCR was done essentially as for 3' RACE<sup>24</sup>, except that after first strand synthesis a primer pair (25 pmol each) specific for either *Sbx* or *Sby* was added. The cycle parameters used were 95 °C (5 min); 72 °C to add 1.25 units of *Taq* polymerase (Perkin-Elmer Cetus); 60 °C (30 s); 72 °C (90 s); and then 95 °C (30 s); 60 °C (30 s); 72 °C (30 s) for 34 cycles followed by 72 °C (15 min). The *Sbx* primers were oMJ41: 5'-TGTCACACCACTTACT-3' and oMJ42: 5'-GCACCTCTGCAACTCCTGG-3'. The *Sby* primers were oMJ13: 5'-CCTCCTAGTCGATGTC-3' and oMJ48: 5'-GACCCCAAGTTCATGGAG-3'. The first strand reaction, containing 3–7 µg total RNA, was diluted to 1 ml and 0.5 µl used for the amplification. For the controls, 50 ng genomic DNA was amplified. Amplified products were electrophoresed on a 2% Nusieve agarose gel in 1 × TAE.

only 95–98% identical to those from the *B3*, *ZfB* or *Zf2* DNA. The *Sbx* cDNA, pCR5 is 1,603 nucleotides long with an ORF across nucleotides 3–1,359. Both cDNAs have poly(A) tails at their presumed 3' ends. They only share homology in the ORFs, where they are 83% identical at the nucleotide level and 85% identical and 92% similar (85/92% id/sim) at the amino-acid level. Database searches revealed that *Sbx* and *Sby* are highly homologous with the human ubiquitin-activating enzyme (E1)<sup>11</sup> and the almost identical A1S9 human cDNA which complements defective DNA replication in the tsA1S9 mutant cell line<sup>15</sup>. At the amino-acid level, *Sby* is 84/92% id/sim to the human E1/A1S9 and *Sbx* is 97/98% id/sim. *Sby* and *Sbx* are also homologous with other cloned E1 genes from wheat<sup>16</sup> and yeast<sup>17</sup> (Fig. 3). As A1S9 and human E1 are virtually identical, A1S9 is probably E1 and *Sbx* represents the mouse homologue of the E1/A1S9 gene. As A1S9 has been localized to Xp11.2-4 in the human<sup>18</sup> the mapping of an *Sbx* homologue to human Xp11.2 (Fig. 2b) is consistent with this interpretation.

The expression patterns of *Sbx* and *Sby* were analysed using the reverse transcriptase-dependent polymerase chain reaction assay (RT-PCR). The results show that *Sbx* is expressed in all male and female tissues tested, consistent with its predicted housekeeping role as a ubiquitin-activating enzyme (Fig. 4). *Sby* expression is testis-specific and its absence in XXSxr<sup>a</sup> testes, which lack germ cells<sup>6</sup>, implies that *Sby* expression is germ-cell dependent. This excludes *Sby* as a candidate for *Hya* as H-Y antigen is known to be expressed in virtually all male tissues.

*Sby* shows good homology to the human ubiquitin-activating enzyme and the (probably identical) A1S9 cDNA both of which have been shown to be essential to normal cell-cycle progression<sup>12,15</sup>. The loss of *Spy* function in the testis of XXSxr<sup>b</sup> mice has been similarly correlated with a reduced mitotic index in the T1 prospermatogonia and the differentiating A spermatogonia<sup>7</sup>. *Sby* may thus encode a testis-specific Y chromosomal E1 enzyme acting to augment insufficient levels of *Sbx* expression in the spermatogonial stages of spermatogenesis. Alternatively, *Sby* could control a specific ubiquitin-dependent pathway in spermatogenesis. To define further its function it

will be important to determine whether *Sby* has ubiquitin-activating properties and if it can complement the loss of function in mutant E1 cell lines. Its map position in the *Sxr<sup>b</sup>* deletion, its testis-specific expression, and its homology with the E1 enzyme are all consistent with *Sby* being *Spy*. Definitive proof of this, however, can only be obtained by reintroducing *Sby* into XXSxr<sup>b</sup> mice to see if it can complement the deleted *Spy* function. □

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## A candidate spermatogenesis gene on the mouse Y chromosome is homologous to ubiquitin-activating enzyme E1

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**THE human X-linked gene A1S9 (refs 1–3) complements a temperature-sensitive cell-cycle mutation in mouse L cells<sup>4</sup>, and encodes the ubiquitin-activating enzyme E1 (refs 5–7). The gene has been reported to escape X-chromosome inactivation<sup>8</sup>, but there is some conflicting evidence<sup>9</sup>. We have isolated part of the mouse A1S9 gene, mapped it to the proximal portion of the X chromosome and shown that it undergoes normal X-inactivation. We also**

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**FIG. 1** Genetic mapping of the mouse *A1s9X* gene. *a*, Hybridization of the 1.5-kb cDNA to a Southern blot of *TaqI*-restricted DNA from *M. musculus* and *M. spretus* revealed restriction fragment length variants between *musculus* (3.4, 2.4 doublet and 1.0 kb) and *spretus* (4.5, 2.0 and 0.5 kb) (left panel). The segregation of these variants was analysed through a mapping panel of 240 interspecific backcross progeny which had been extensively characterised for X-chromosome markers<sup>15</sup>. Examination of individual male and female backcross progeny (right panel) revealed clear male-specific bands (18, 9, 7.9, 4.6, 3.4, 2.8, 1.6 and 1.2 kb) indicative of homologous sequences on the Y chromosome. Molecular sizes are indicated in kb. *b*, Interspecific backcross pedigree analysis of the 240 progeny shows that the *A1s9X* gene maps to the proximal region of the mouse X chromosome, between *Otc* and *Hprt*. Genetic distance between loci is indicated in centiMorgans  $\pm$  s.e.; the figure in brackets indicates recombination fractions between adjacent pairs of loci.

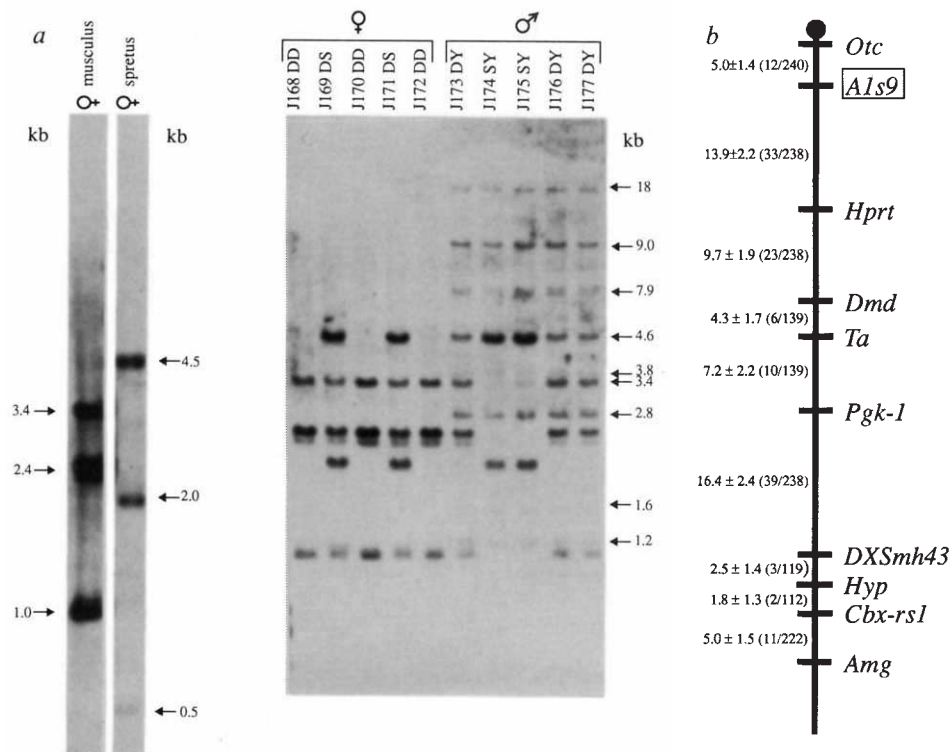
**METHODS.** The 1.5 kb mouse *A1s9X* probe was cloned using PCR from mouse liver cDNA and primers TTCATGCCCAT(T,C)ATGCA-(G,A)TGG and AAGCGATCCCACA(A,G)(T,G)-GTCCA, corresponding to nucleotides 544–564 and 2,168–2,149 of the human *A1S9* sequence<sup>9</sup>. Southern hybridizations were done under standard conditions<sup>11</sup>. The production of the interspecific backcross progeny and the details of the characterization of their X

detected two copies of the gene on the short arm of the mouse Y chromosome (*A1s9Y-1* and *A1s9Y-2*). The functional *A1s9Y* gene (*A1s9Y-1*) is expressed in testis and is lost in the deletion mutant *Sxr<sup>b</sup>* (ref. 10). Therefore *A1s9Y-1* is a candidate for the spermatogenesis gene, *Spy*, which maps to this region. *A1s9X* is similar to the *Zfx* gene in undergoing X-inactivation<sup>11,12</sup>, yet having homologous sequences on the short arm of the Y chromosome<sup>13,14</sup>, which are expressed in the testis. These Y-linked genes may form part of a coregulated group of genes which function during spermatogenesis.

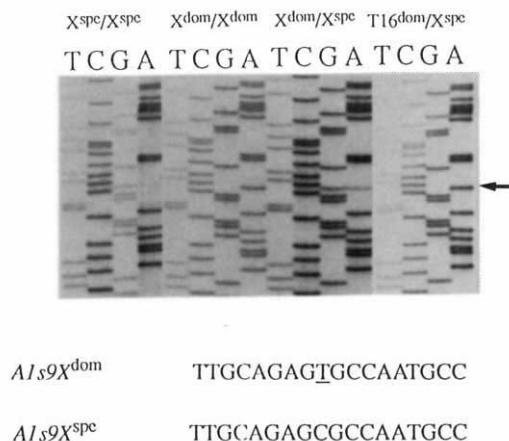
We generated at 1.5-kilobase (kb) mouse complementary DNA probe using reverse transcriptase polymerase chain reaction (RT-PCR) with primers made from the published human *A1S9* sequence<sup>9</sup>. To map the *A1s9* gene on the mouse X chromosome, *Mus musculus*/*Mus spretus* interspecific backcross pedigree analysis was used. The *A1s9X* probe detected *TaqI* restriction fragment length variants between *musculus* and *spretus* (Fig. 1*a*, left). The segregation of these variants was analysed through a panel of 240 interspecific backcross progeny, previously characterized with molecular and genetic markers along the length of the X chromosome<sup>15–17</sup>. The *A1s9X* gene maps to the position shown in Fig. 1*b* between *Otc* and *Hprt*, in the region predicted from the known syntenic relationship between the human and mouse X chromosomes<sup>18</sup>.

To determine whether *A1s9X* undergoes X-chromosome inactivation, interspecific F<sub>1</sub> hybrids between *M. musculus* (dom) carrying the T(X;16)16H (T16<sup>dom</sup>) translocation and *M. spretus* (spe) were used. T16<sup>dom</sup>/X<sup>spe</sup> mice exhibit total nonrandom inactivation of the X<sup>spe</sup> chromosome<sup>11</sup>. Expression of the X<sup>spe</sup> allele of a particular gene in these mice indicates that the locus in question escapes inactivation<sup>17</sup>. Figure 2 shows that T16<sup>dom</sup>/X<sup>spe</sup> mice express only the X<sup>dom</sup> allele of *A1s9X*, which demonstrates that *A1s9X* undergoes X-chromosome inactivation.

Southern analysis of the interspecific backcross mice unexpectedly revealed *A1s9*-homologous sequences on the Y chromosome (Fig. 1*a*, right). This was confirmed by further Southern



chromosomes are described elsewhere<sup>15</sup>



**FIG. 2** *A1s9X* undergoes X-chromosome inactivation in the mouse. RNA prepared from the livers of X<sup>spe</sup>/X<sup>spe</sup>, X<sup>dom</sup>/X<sup>dom</sup>, X<sup>dom</sup>/X<sup>spe</sup> and T16<sup>dom</sup>/X<sup>spe</sup> mice was reverse-transcribed into cDNA. *A1s9X* cDNA was amplified using gene-specific primers and the PCR products directly sequenced. *A1s9X<sup>spe</sup>* and *A1s9X<sup>dom</sup>* differ by a sequence variant at the position indicated by the arrowhead. The control interspecific F<sub>1</sub> animal (X<sup>dom</sup>/X<sup>spe</sup>) expresses both alleles, yet the T16<sup>dom</sup>/X<sup>spe</sup> mouse expresses only the *A1s9X<sup>dom</sup>* allele, demonstrating that *A1s9X* undergoes X-inactivation.

**METHODS.** X<sup>spe</sup>/X<sup>spe</sup>, X<sup>dom</sup>/X<sup>dom</sup>, X<sup>dom</sup>/X<sup>spe</sup> and T16<sup>dom</sup>/X<sup>spe</sup> mice were derived as described previously<sup>11</sup>. RNA was prepared from liver using the guanidinium thiocyanate method or using a FastTrack kit (Invitrogen). cDNA was synthesized with oligo dT as primer, amplified using PCR and sequenced directly as described<sup>11</sup>. *A1s9X* primers GAACCGACGTTTCTCTCC and CAGCCACAATGAAATCCATG were derived from the sequence of *A1s9X* cDNA (see legend to Fig. 1) and amplify a 1.0 kb fragment. Control PCR reactions on RNAs incubated without reverse transcriptase demonstrated that amplification was not due to contaminating genomic DNA. Sequence variants between *M. musculus* and *M. spretus* were identified by DNA sequencing. The *A1s9X* sequence given is the coding strand and is complementary to that shown on the sequencing gel.

analysis (Fig. 3a). These *A1s9Y* sequences were mapped to the short arm of the Y chromosome by Southern analysis of  $XXSxr^a$  and  $XXSxr^b$  mice (Fig. 3b).  $Sxr^a$ , sex-reversed<sup>19-23</sup> (formerly called *Sxr*) is a mutation caused by the transfer to the X chromosome of a small part of the Y chromosome short-arm material, including the testis-determining gene<sup>24</sup>.  $Sxr^b$ , formerly called *Sxr'* (ref. 10), is a variant of  $Sxr^a$  that has deleted some of the Y chromosomal material, but which has retained the testis-determining gene. All the *A1s9Y* sequences are present in  $XXSxr^a$  animals (Fig. 3b). Most of the Y-specific sequences are also present in  $XXSxr^b$ . But a single band was deleted in *TaqI* and *BamHI* digests of  $XXSxr^b$  DNA (Fig. 3b). Even allowing for loading differences, certain bands in *TaqI*, *BamHI* and *MspI* digests were also much weaker in  $XXSxr^b$  compared with  $XXSxr^a$  (Fig. 3b), suggesting the presence of two or more genes, at least one of which is lost in  $Sxr^b$ . A cosmid containing part of the *A1s9Y* gene deleted in  $Sxr^b$  (*A1s9Y-1*) was isolated and partially sequenced (Fig. 4a), showing that *A1s9Y-1* encodes a protein homologous to that encoded by *A1s9X*. The sequence of the corresponding region of *A1s9Y-2*, the gene present in  $Sxr^b$ , reveals that it is a pseudogene containing an in-frame termination codon (Fig. 4b) that would result in the production of a nonfunctional protein.

Although *A1s9X* is ubiquitously expressed (Fig. 4c, bottom), *A1s9Y-1* is only expressed significantly in testis (Fig. 4c, top), although a small amount of expression is seen in brain. Expression of *A1s9Y-2* was not detected in testis (data not shown). The *A1s9Y-1* gene is transcribed in  $XXSxr^a$  testis but not in  $XXSxr^b$  testis (Fig. 4d).

*A1s9Y-1* may encode one of the two functionally defined genes, *Hya* (ref. 25) and *Spy* (ref. 26), deleted in  $Sxr^b$ . The restricted pattern of *A1s9Y-1* expression excludes the possibility

that it is *Hya*, which encodes the male-specific H-Y antigen. But the testis-specific expression of *A1s9Y-1* makes it a candidate for *Spy*, a gene necessary for spermatogonial proliferation which is blocked in  $XXSxr^b/0$  but not in  $XXSxr^a/0$  animals<sup>26</sup>. One would predict that the *Spy* gene product would probably be expressed in germ cells.  $XXSxr^a$  and  $XXSxr^b$  testes have very few germ cells as an XX chromosome constitution is detrimental to male germ-cell viability (reviewed in ref. 27). Detection of *A1s9Y* expression in  $XXSxr^a$  testes, despite the paucity of germ cells, may reflect the sensitivity of the PCR assay. Alternatively, *A1s9Y-1* may be expressed in the somatic supporting-cell component of the testis.

The nature of the *A1s9Y* gene product is consistent with an essential role in spermatogenesis. *A1s9* encodes the ubiquitin-activating enzyme E1 (*UBA1*)<sup>5-7</sup>, which catalyses the first step in ubiquitin-mediated protein degradation in eukaryotic cells (reviewed in ref. 28) and a functional *UBA1* gene is necessary for cell viability<sup>5</sup>. The absence of *A1s9Y-1* expression in  $XXSxr^b/0$  germ cells may block proliferation, which would be analogous to the cell-cycle arrest in cells carrying the temperature-sensitive *tsA1S9* mutation<sup>4</sup>. Although premeiotic  $XXSxr^b/0$  germ cells should still express *A1s9X*, it is possible that a boost in expression, provided by *A1s9Y-1*, is necessary for successful spermatogonial proliferation. As selective protein degradation is likely to be particularly important during spermatogenesis, it is possible that *A1s9Y-1* expression could also functionally substitute for *A1s9X* when the X chromosome is inactivated at the pachytene stage of male meiosis<sup>29</sup>.

The other formal candidate for *Spy*, *Zfy-2* (ref. 30), which is deleted in  $Sxr^b$ , also has expression restricted to male germ line and an X-linked homologue which undergoes X-inactivation<sup>11,12</sup>. As the *A1s9Y* and *Zfy* genes are linked on the short

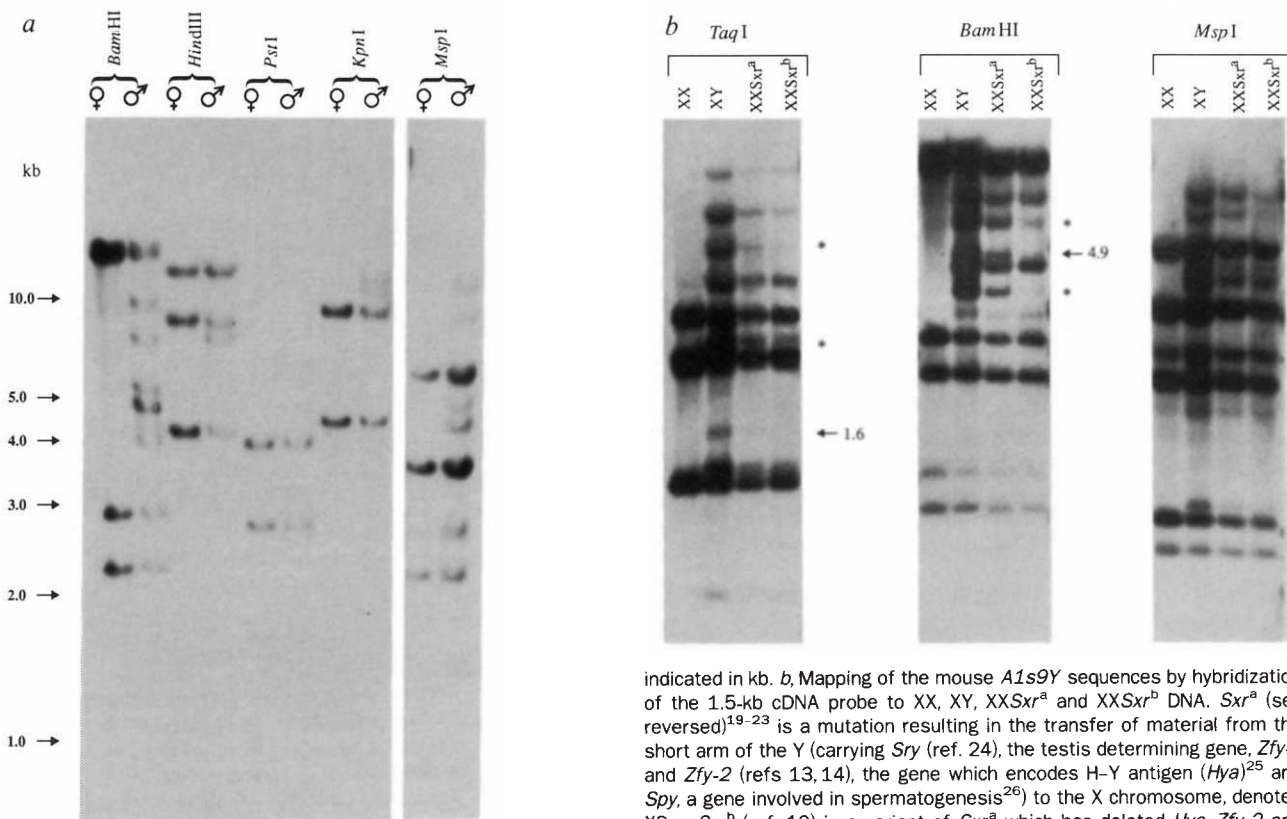
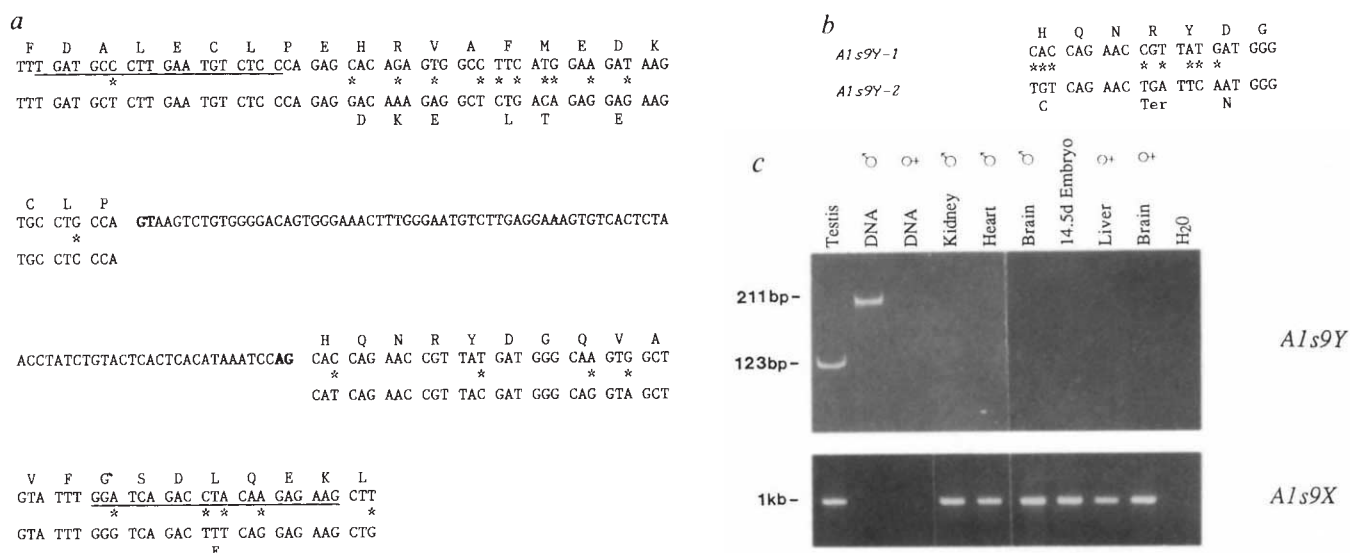


FIG. 3 Homologous *A1s9Y* sequences on the short arm of the mouse Y chromosome. *a*, Hybridization of the 1.5-kb mouse cDNA probe to male and female DNA digested with a number of different restriction enzymes. Clear male-specific bands are seen with all the enzymes, confirming the presence of homologous sequences on the Y chromosome. Molecular sizes are

indicated in kb. *b*, Mapping of the mouse *A1s9Y* sequences by hybridization of the 1.5-kb cDNA probe to XX, XY,  $XXSxr^a$  and  $XXSxr^b$  DNA.  $Sxr^a$  (sex reversed)<sup>19-23</sup> is a mutation resulting in the transfer of material from the short arm of the Y (carrying *Sry* (ref. 24), the testis determining gene, *Zfy-1* and *Zfy-2* (refs 13, 14), the gene which encodes H-Y antigen (*Hya*)<sup>25</sup> and *Spy*, a gene involved in spermatogenesis<sup>26</sup>) to the X chromosome, denoted  $XSxr$ .  $Sxr^b$  (ref. 10) is a variant of  $Sxr^a$  which has deleted *Hya*, *Zfy-2* and *Spy* (refs 13, 26). Restriction fragments deleted in  $XXSxr^b$  are indicated with arrows; restriction fragments showing dosage differences in  $XXSxr^b$  and  $XXSxr^a$  are indicated by asterisks. Molecular sizes are indicated in kb. METHODS.  $XXSxr^a$  and  $XXSxr^b$  mice were offspring from breeding colonies of mice carrying  $Sxr^a$  and  $Sxr^b$  maintained at the Clinical Research Centre. Southern hybridizations were done under standard conditions<sup>11</sup>.





**FIG. 4** Expression of *A1s9X* and *A1s9Y* genes. **a**, Structure of part of the *A1s9Y-1* gene. Sequence of a region of the *A1s9Y*-locus (upper line) containing a small intron is shown compared with that of *A1s9X* cDNA (lower line), differences being indicated by asterisks. Consensus splice donor and acceptor sites are shown in bold. The amino acids encoded by *A1s9Y-1* and differences in *A1s9X* are indicated in one letter amino-acid code. Sequences to which PCR primers were constructed are underlined. **b**, *A1s9Y-2* is a pseudogene, containing an in-frame termination codon. The *A1s9Y-2* gene was amplified from XXSx<sup>b</sup> DNA using *A1s9Y*-specific PCR primers and sequenced directly. The sequence of *A1s9Y-2* and *A1s9Y-1* adjacent to the splice acceptor site (bold in Fig. 4a) is shown. The termination codon present in *A1s9Y-2* would result in a truncated protein product of about one-third the normal length<sup>5</sup>. **c**, PCR analysis of *A1s9Y-1* and *A1s9X* expression. Reverse-transcribed RNAs and genomic DNA from male and female mice were amplified with primers specific for *A1s9Y* (upper panel) or *A1s9X* (lower panel). The specificity of the primers for *A1s9Y* under the conditions used is indicated by the absence of amplification from the female samples. Significant expression of *A1s9Y* is restricted to the testis in males, although there is a lower level of expression in the brain. Sequence analysis of the testis-expressed PCR product indicates that *A1s9Y-2* is not expressed. *A1s9X* cDNA is efficiently amplified from both male and female samples (bottom). The 14.5-day embryos were a mixture of males and females. **d**, *A1s9Y-1* is not expressed in testis of XXSx<sup>b</sup> mice. Analysis of *A1s9Y-1* expression was as in **c**. *A1s9Y-1* is efficiently amplified from the testis of XXSx<sup>b</sup> mice, despite the very low number of germ cells present. There is no expression of *A1s9Y-1* in XXSx<sup>b</sup> testis.

**METHODS.** A genomic *A1s9Y* clone was isolated by screening a male mouse

cosmid library with the *A1s9X* cDNA clone (see legend to Fig. 1). The cosmid was digested with *Hind*III and ligated to *Hind*III-cut pBluescript. The ligation was then included in a PCR with *A1s9X*- and pBluescript-specific primers. Amplified bands were directly sequenced to identify sequences for Y-specific PCR primers. RNA was prepared and reverse transcribed as in Fig. 2. *A1s9Y*-specific primers (TGATGCCCTTGAATGTCTCC and CTTCTCTGTAGGCTCTGATCC) amplify a 123-base-pair (bp) fragment from cDNA and one of 211 bp from genomic DNA. These fragments were resolved on a 10% polyacrylamide gel. *A1s9X* primers were as described in Fig. 2; PCR products were resolved on 1.2% agarose gels.

arm of the Y chromosome they may be part of a coregulated group of genes required during spermatogenesis. One might expect such functions to be conserved in mammals. Indeed, there are homologous Y-linked *A1s9* sequences in the rat (A.A.,

unpublished results) but no evidence for homologous sequences on the human Y. Nevertheless, it remains possible that there is an evolutionarily diverged Y-linked or autosomal copy in humans. □

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# A molecular chaperone from a thermophilic archaeobacterium is related to the eukaryotic protein t-complex polypeptide-1

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**THERE** is evidence to suggest that components of archaeobacteria are evolutionarily related to cognates in the eukaryotic cytosol<sup>1–7</sup>. We postulated that the major heat-shock protein of the thermophilic archaeobacterium, *Sulfolobus shibatae*, is a molecular chaperone and that it is related to an as-yet unidentified chaperone component in the eukaryotic cytosol. Acquired thermotolerance in *S. shibatae* correlates with the predominant synthesis of this already abundant protein, referred to as thermophilic factor 55 (TF55; ref. 8). TF55 is a homo-oligomeric complex of two stacked 9-membered rings, closely resembling the 7-membered-ring complexes of the chaperonins, groEL, hsp60 and Rubisco-binding protein<sup>9–15</sup>. The TF55 complex binds unfolded polypeptides *in vitro* and has ATPase activity—features consistent with its being a molecular chaperone<sup>16,17</sup>. The primary structure of TF55, however, is not significantly related to the chaperonins. On the other hand, it is highly homologous (36–40% identity) to a ubiquitous eukaryotic protein, t-complex polypeptide-1 (TCP1; refs 18–20). In *Sac-*

*charomyces cerevisiae*, TCP1 is an essential protein that may play a part in mitotic spindle formation<sup>21</sup>. We suggest that TF55 in archaeobacteria and TCP1 in the eukaryotic cytosol are members of a new class of molecular chaperones.

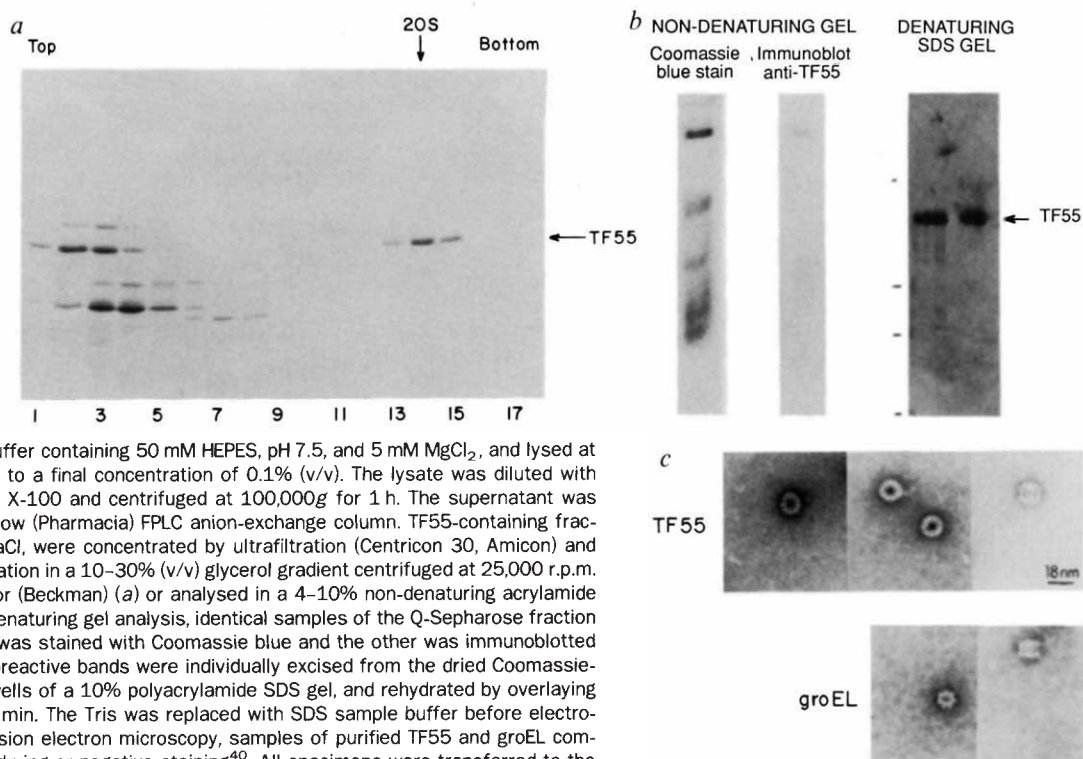
TF55 was purified from the soluble portion of Triton X-100 cell extracts by anion exchange chromatography and glycerol gradient sedimentation. Most of the TF55 sedimented in the gradient at 20S (Fig. 1a). No nucleic acid was found by spectrophotometry in the 20S fraction, suggesting that TF55 is not part of a ribonucleoprotein complex. SDS-polyacrylamide gel electrophoresis of this fraction showed that it contained only TF55 protein, indicating that it is a homo-oligomeric complex. Similar sedimentation behaviour is exhibited by the chaperonins groEL, hsp60 and Rubisco-binding protein, which are 14-mer complexes<sup>9–11,15,22</sup>. In a non-denaturing polyacrylamide gel, TF55 migrates more slowly than the chaperonins<sup>23–25</sup>, and as two bands instead of one (Fig. 1b, lanes 1 and 2). When these bands were excised and analysed by SDS-PAGE, both ran with an apparent relative molecular mass of 55,000 ( $M_r$  55K) (Fig. 1b, lanes 3 and 4).

In scanning transmission electron micrographs, the TF55 complex usually appears in negative stain as a nine-unit, radially symmetric ring with a dark core in one orientation (end view), and a 2-fold symmetrical layered structure with connecting 'fingers' in the other (side view) (Fig. 1c, upper panel). In comparison, groEL has a similar double-ring structure, but with an apparently 7-fold symmetry when viewed end-on and a more flattened appearance from the side (Fig. 1c, lower panel). Images of negatively stained TF55 complex show that most end views have 9-fold symmetry, but some 8-fold symmetrical complexes are also seen. Diameter and  $M_r$  determinations of unstained freeze-dried specimens in the scanning transmission electron microscope also indicate that there may be two size classes. The  $M_r$  of complexes with a smaller radius (<12.1 nm) was  $930 \pm 50K$  ( $n=32$ ), and  $M_r$  for the larger complexes was  $1,000 \pm 64K$  ( $n=130$ ), values that would be expected for complexes with

**FIG. 1** Purified TF55 is a homo-oligomeric double ring complex. **a**, TF55 sediments at 20S in a glycerol gradient. **b**, TF55 migrates as a doublet in a non-denaturing polyacrylamide gel. Migration positions of molecular size markers in SDS-PAGE are indicated from top to bottom: 66K, 45K, 36K, 29K. **c**, TF55 appears as a stacked ring complex in scanning transmission electron microscopy. Scale bar, 18 nm.

**METHODS.** *S. shibatae* cells were grown at 75°C as described<sup>8</sup>, then collected,

washed and resuspended in a buffer containing 50 mM HEPES, pH 7.5, and 5 mM  $MgCl_2$ , and lysed at 4°C by addition of Triton X-100 to a final concentration of 0.1% (v/v). The lysate was diluted with the buffer to 0.01% (v/v) Triton X-100 and centrifuged at 100,000g for 1 h. The supernatant was applied to a Q-Sepharose fast-flow (Pharmacia) FPLC anion-exchange column. TF55-containing fractions, eluted at 150–200 mM NaCl, were concentrated by ultrafiltration (Centricon 30, Amicon) and further fractionated by sedimentation in a 10–30% (v/v) glycerol gradient centrifuged at 25,000 r.p.m. for 20 h at 4°C in an SW27 rotor (Beckman) (**a**) or analysed in a 4–10% non-denaturing acrylamide gel (**b**) as described<sup>23</sup>. For non-denaturing gel analysis, identical samples of the Q-Sepharose fraction were run in two lanes; one lane was stained with Coomassie blue and the other was immunoblotted as described<sup>24</sup>. The two immunoreactive bands were individually excised from the dried Coomassie-stained gel, placed in adjacent wells of a 10% polyacrylamide SDS gel, and rehydrated by overlaying with 1 M Tris-HCl, pH 8.0, for 60 min. The Tris was replaced with SDS sample buffer before electrophoresis. For scanning transmission electron microscopy, samples of purified TF55 and groEL complex<sup>39</sup> were prepared by freeze-drying or negative-staining<sup>40</sup>. All specimens were transferred to the microscope under vacuum and observed at –150°C with minimal dose techniques. Scattered electron counts were recorded digitally as the 0.25-nm probe (40 keV) scanned the specimen in a 512 × 512 raster.  $M_r$  measurements on the unstained, freeze-dried specimens were made as before<sup>40</sup>. Particles for  $M_r$  analysis were selected on the basis of their separation from neighbours, clean surrounding background, sharp edges and lack of obvious defects.







**a** -95 5 - CATATTTCGAACTACGAGAAAGTATGGATGCTGATTTGAGCAAAATTTTATAACCTTTTAAAGCAGAGTGGAGGCGCTAAA

1 ATG GCA ACA GGT ACA GTT GCA ACT ACA CCC GAA GGT ATA CCT GTA ATA ATT TTA AAA GAG GGA TCA AGT AGA ACA  
Met Ala Thr Ala Thr Val Ala Thr Thr Pro Glu Gly Ile Pro Val Ile Ile Leu Lys Gly Ser Ser Arg Thr 25

76 TAT GGA AAA GAA GCT TTA AGC GGT AAT ATT GCT GCA GTG AAA GCA ATT GAA GAG GCA TTA AAA AGC ACC TAT GGT  
Tyr Gly Lys Glu Ala Leu Arg Ala Asn Ile Ala Ala Val Lys Ile Glu Glu Ala Leu Lys Ser Thr Tyr Gly 50

151 CCA GGT GGA ATG GAT AAG ATG TTC GTC GAT AGC TTA GGA GAT ATT ACA ATA ACA AAT GAT GGA GCC ACT ATT GCT  
Pro Arg Gly Met Asp Lys Met Pro Val Asp Ser Leu Gly Asp Ile Thr Ile Thr Asn Asp Gly Ala Thr Ile Leu 75

226 GAT AAA ATG GAT TTA CAA CAC CCA ACA GGT AAG GCT TTA GTT CAG ATA GCT AAA GGA CAA GAG GAG GAA ACA GCT  
Asp Lys Met Asp Leu Gln His Pro Thr Gly Lys Leu Leu Val Gln Ile Ala Lys Gly Gln Asp Glu Glu Thr Ala 100

301 GAT GGC ACT AAA ACT GCT GTA ATT CTT GCT GGA GAA TTA GCT AAA AAA GCA GAA GAT CTT TTA TAT AAG GAG ATT  
Asp Gly Thr Lys Thr Ala Val Ile Leu Ala Gly Glu Leu Ala Lys Lys Ala Glu Asp Leu Leu Tyr Lys Glu Ile 125

376 CAC CCA ACA ATA ATT GTA AGC GGA TAT AAG AAG CCA GAA GAT ATT GCA TTA AAG ACC AIC CAA GAT ATA GCA CAA  
His Pro Thr Ile Ile Val Ser Gly Tyr Lys Lys Ala Glu Glu Ile Ala Leu Lys Thr Ile Gln Asp Ile Ala Gln 150

451 CGG GTC AGC ATA AAT GAT ACT GAT GCA CTT AGG AAA GCA GCA TTA ACA TCC TTA GGC AGT AAG GCA GTA GCA GGC  
Pro Val Ser Ile Asn Asp Thr Asp Val Leu Arg Lys Val Ala Leu Thr Ser Leu Gly Ser Lys Ala Val Ala Gly 175

526 GCA GGA GAG TAT TTA GCT GAC CTT GTG GTT AAA GCA GTG GCA CAA GAA TTA AGA GGA GAT AAG TGG TAT  
Ala Arg Glu Tyr Leu Ala Arg Leu Val Lys Lys Ala Val Ala Gln Val Ala Glu Leu Arg Gly Asp Lys Thr Tyr 200

601 GTT GAT CTA GAT AAT GCA CAA ATT AAA AAA CAT GGT GGT AAT GAT ACT CCA TTA TTA CAC GGC ATC  
Val Asp Leu Asp Asn Val Gln Ile Val Lys Lys His Gly Gly Ser Ile Asn Asp Thr Gln Leu Val Tyr Gly Ile 225

676 GTA GTT GAT AAG GAA GGT GAT CAT CCG GGC ATG CCA AAG AAG ATT GAA AAT GCT AAG ATA GGC CTT TTA GAC GCT  
Val Val Asp Lys Glu Val Val His Pro Gly Met Pro Lys Arg Ile Glu Asn Ala Lys Ile Ala Leu Leu Asp Ala 250

751 TCA TTA GAA GGT GAG AAA CCC GAA TTG GAT GCA GAA ATA AGA ATT AAC GAT CCA ACA CAG AGC CAC AAA TTC TTG  
Ser Leu Glu Val Glu Lys Pro Glu Leu Asp Ala Glu Ile Asn Asp Thr Thr Gln Met His Lys Phe Leu 275

826 GAA GAA GAA GAA AAC ATA TTG AAA GAA AAA GTA GAT AAG ATT GCA GCT ACT GGT GCT AAG GTC ATA TCC CAC  
Glu Glu Glu Glu Asn Ile Leu Lys Glu Lys Val Asp Lys Ile Ala Ala Thr Gly Ala Asn Val Val Ile Cys Gln 300

901 AAA GGT ATC GAT GAA GTT GCA CAA CAC TAT TTA GCT AAG AAA GGT ATA TTA GCT GTT AGG AGA GCC AAG AGT  
Lys Gly Ile Asp Glu Val Ala Gln His Tyr Leu Ala Lys Lys Gly Ile Leu Ala Val Arg Arg Ala Lys Lys Ser 325

976 GAT TTA CAG AAA TTA GCT AGA GGT ACC GGA GGT AGA GTC ATA TCA AAT ATT GAT GAA TTA ACT TCA CAA GAT CTA  
Asp Leu Glu Lys Leu Ala Arg Ala Thr Gly Arg Val Ile Ser Asn Ile Asp Glu Leu Thr Ser Gln Asp Leu 350

1051 GGT TAT GGC GCA TTA GTG GAA GAG ACA AAA GAA GGA GAT AAG AGG GTA TTC GTA GCA GGT CCA AAG AAT CCA  
Gly Tyr Ala Ala Leu Val Glu Glu Arg Lys Val Gly Glu Asp Lys Met Val Phe Val Glu Gly Ala Lys Asn Pro 375

1126 AAA TCA GGT AGT ATA CTA ATA AGA GGA GGA TTA GAG GTC ATA GAT GAT GAT CCA AAG GAT CTT AGG GAC GCT  
Lys Ser Val Ser Ile Leu Ile Arg Gly Gly Leu Glu Arg Val Val Asp Glu Thr Glu Arg Ala Leu Arg Asp Ala 400

1201 TTA GGT ACA CTG CCA GAT GAT ATA AGG GAT GGT AGA GCA GCA GCT GGT GGT GCA GCT GGT GAT ACA GAT ATA GCT  
Val Ile Glu Thr Val Ala Leu Val Ile Arg Asp Gly Arg Ala Val Ala Gly Gly Arg Val Ala Leu Glu Ile Ala 425

1276 AAG AGA TTA AGA AAG TAT GGC CCA CAA GTT GGT GGT AAA GAG CAA TTA GCA ATT GAA GCA TAT GCT AAT CCA ATA  
Lys Arg Leu Arg Lys Tyr Ala Val Glu Gly Lys Glu Gln Leu Ala Ile Glu Ala Tyr Ala Asn Ala Ile 450

1351 GAA GGA CTT ATC ATG ATA TTG GCG GAA AAC GCA GCA TTA GAT CTT ATA GAC AAA TTA ATG CAA TTA AGA AGT CTT  
Glu Gly Leu Ile Met Ile Leu Ala Glu Asn Ala Gly Leu Asp Pro Ile Asp Lys Leu Met Gln Leu Arg Ser Leu 475

1426 CAC GAG AAT GAG ACC AAT AAA TGG TAT GCA CTT AAT TTA TTT ACT GGA AAT CCA GAG GAT ATG TGG AAA TTA GGT  
His Glu Asn Glu Thr Asn Lys Trp Tyr Cys Leu Asn Leu Phe Thr Gly Asn Pro Glu Asp Met Trp Lys Leu Gly 500

1501 GTT ATT GAA CCG GCA CTA GTG AAA ATG AAT GCA ATT AAC GCT ACA ACA GAA GCA GTA ACA TTA GTG TTA AGA ATA  
Val Ile Glu Pro Ala Leu Val Lys Met Asn Ala Ile Lys Ala Ala Thr Glu Ala Val Thr Leu Val Leu Arg Ile 525

1576 GAT GAT ACT GTA GCA GCT GGA AAG AAG GGT GCA AGT GAG CCA GGC GGT AAG AAA GAA AAA GAA AAG TCC TCT  
Asp Asp Ile Val Ala Ala Gly Lys Lys Gly Gly Ser Glu Pro Glu Gly Lys Glu Lys Glu Lys Glu Lys Glu 550

1601 GAA GAC TAAAGCAATT TTTAATTTT AATTATATA GGAACCTTA GATTGGGA TCGCTTATTA CAGC/CGCC TATGCTAGI - 3'  
Glu Asp

**b**

0 10 20 30 40 50

Mouse TCP1  
YF55  
Yeast TCP1

1 MEGLPLSVFGDRST-GEA--VRSQNVMAAASIA--NIV--KSS  
2 MATAATVATTPPEGIPV--LKEGSSRYGKEALANIAAVKAIIEALKST  
3 MSQCFNNRSRSTLFLGGKISGGDD--NQNVLATMAVANV--KSS

48

MOUSE TCP1  
YF55  
YEAST TCP1

4 FCPVGLDKMVDIDGDTITNDGATIKLLEVEHPAAKVLCLLADLQDKE  
5 YGPRAGMDKMFVDSLGDTITNDGATIKLLEVEHPAAKVLCLLADLQDKE  
6 LGPVGLDKMVDIDGDTITNDGATIKLLEVEHPAAKVLCLLADLQDKE

98

MOUSE TCP1  
YF55  
YEAST TCP1

7 VGDGTTSVVTIAAELLKNADELVKQKIHPTSVISGYRLACKFAVRYINEN  
8 TADGCTKIAVTVIRKKAEDLLYKEIHPTSVISGYRLACKFAVRYINEN  
9 VGDGTTSVVTIAAELLKNADELVKQKIHPTSVISGYRLACKFAVRYINEN

147

MOUSE TCP1  
YF55  
YEAST TCP1

10 LIINIVDELGRDCLINIAKTSMSKKIICINGDYFANMVVDVLA VKYTQAR  
11 IAQFVSLINDTVIRKKAEDLLYKEIHPTSVISGYRLACKFAVRYINEN  
12 LSTSVDTLCKETLINIAKTSMSKKIICADSDFFSNMVVDVLA VKYTQNSK

195

MOUSE TCP1  
YF55  
YEAST TCP1

13 GQPRPV--PVNSVNIIVKAGHRSQIESVILINGYALNVCVSGQMPKRI--VNA--  
14 GCKVYVLDLNVQVIVKAGHRSQIESVILINGYALNVCVSGQMPKRI--VNA--  
15 SEITPV--PVKAVNVLKAGHRSQIESVILINGYALNVCVSGQMPKRI--VNA--

243

MOUSE TCP1  
YF55  
YEAST TCP1

16 KTAACLDL--S--QKTKMKELGVQVVIITDFEKLDCIQRRESDITKRIQKILATG  
17 KTAACLDL--S--QKTKMKELGVQVVIITDFEKLDCIQRRESDITKRIQKILATG  
18 KTAACLDL--S--QKTKMKELGVQVVIITDFEKLDCIQRRESDITKRIQKILATG

293

MOUSE TCP1  
YF55  
YEAST TCP1

19 AXVLLITGCTGMYLKFEVAGAMAVRRVLRNODKHVAVRALSASILSTLA  
20 ANVVICGKGDSVAQHILAKKCLILVRRARAKSDLEKLARATGGRVISNID  
21 AGVLLITGCTGMYLKFEVAGAMAVRRVLRNODKHVAVRALSASILSTLA

343

MOUSE TCP1  
YF55  
YEAST TCP1

22 NLEGEFTFEVMTLSQAEVVOERICDDELIIKHTKARTASISILIRGAND  
23 NLEGEFTFEVMTLSQAEVVOERICDDELIIKHTKARTASISILIRGAND  
24 NLEGEFTFEVMTLSQAEVVOERICDDELIIKHTKARTASISILIRGAND

387

MOUSE TCP1  
YF55  
YEAST TCP1

25 FMCDEMERSTHDAICVVKRVLELKSVPVCGGAVAAALSIIYENYATNMCS  
26 RVVDETERARDAALGTVAQVIRGAVVCGGAVAAALSIIYENYATNMCS  
27 YSLDEMERSTHDAICVVKRVLELKSVPVCGGAVAAALSIIYENYATNMCS

437

MOUSE TCP1  
YF55  
YEAST TCP1

28 RECLAIAEEFAASLLVVPNTLAVNAAGSDTDLVAKLRAAFHNEAQVNPERKN  
29 RECLAIAEEFAASLLVVPNTLAVNAAGSDTDLVAKLRAAFHNEAQVNPERKN  
30 RECLAIAEEFAASLLVVPNTLAVNAAGSDTDLVAKLRAAFHNEAQVNPERKN

480

MOUSE TCP1  
YF55  
YEAST TCP1

31 LK--G--GIDLVHGGKPRDNKKQAGVFEPTIVKVKSLKFAEATATTEBRI  
32 LK--G--GIDLVHGGKPRDNKKQAGVFEPTIVKVKSLKFAEATATTEBRI  
33 LK--G--GIDLVHGGKPRDNKKQAGVFEPTIVKVKSLKFAEATATTEBRI

525

MOUSE TCP1  
YF55  
YEAST TCP1

34 DLIKLHPESKDDKHSSYENAVHSGALCD  
35 DLIKLHPESKDDKHSSYENAVHSGALCD  
36 DLIKLHPESKDDKHSSYENAVHSGALCD

552

MOUSE TCP1  
YF55  
YEAST TCP1

37 DLIKLHPESKDDKHSSYENAVHSGALCD  
38 DLIKLHPESKDDKHSSYENAVHSGALCD  
39 DLIKLHPESKDDKHSSYENAVHSGALCD

**FIG. 3** Nucleotide sequence of *S. shibatae* TF55 gene and comparison of the predicted amino-acid sequence with eukaryotic TCP1. **a**, DNA sequence of the cloned TF55 gene and its predicted amino-acid sequence; **b**, Comparison of the amino-acid sequence of TF55 with that of mouse TCP1b and with TCP1 from *S. cerevisiae*.

**METHODS.** TF55 protein from heat-shocked cells was excised from an SDS-polyacrylamide gel, and the amino-acid sequence of two peptides was determined by sequential Edman degradation (underlined in **a**; peptide 1 corresponds to amino-acid residues 514–524; peptide 2 corresponds to residues 338–359). Degenerate polymerase chain reaction (PCR) primers (23-mers) were constructed, incorporating inosine at 4-base degenerate positions, corresponding to amino acids 1–9 of peptide 1 and amino acids 12–20 of peptide 2. Because the order of the two peptides in TF55 was then unknown, both coding and noncoding strands were synthesized. One of the pairs of primers produced a 520-base-pair product when PCR amplification of genomic *S. shibatae* DNA was carried out (30 cycles at 95 °C for 2 min, 37 °C for 2 min and 67 °C for 2 min). No product was observed with the other pair of primers. The PCR product was cloned in pBluescript (Stratagene). DNA-sequence analysis predicted the remaining 3 residues of peptide 2, not represented in the PCR primer, as well as an additional tryptic peptide that had been previously sequenced (corresponding to amino acids 475–482). The cloned segment was used to screen two libraries constructed from *S. shibatae* genomic DNA digested either with *Pst*I or *Xba*I. Two overlapping clones were sequenced to produce the sequence shown in **a**. The canonical archaeobacterial promoter element (TTTATA) (ref. 3) is underlined. Nucleotides are numbered on the left and amino acids on the right, in both cases from the translational start. The 55K protein recovered by SDS-PAGE and predicted by the DNA sequence was identical to that from the purified complex (Fig. 1). This identity was confirmed by the precise match of the amino-acid sequence determined by sequential Edman degradation of several tryptic peptides prepared from the purified complex with the amino-acid sequence predicted by the DNA sequence in **a**. **b**, A 3-way comparison of sequences (TF55, Fig. 4a; TCP1b mouse, GENBANK M12899 and K. Willis (personal communication); TCP1 *S. cerevisiae*, GENBANK M21160) was carried out by the computer program THREEalign 2.0.



TABLE 1 Sequence comparisons

|            | TCP1<br>mouse | TCP1<br>yeast | groEL | hsp60 | hsp65 | hsp70 | dnaK  |
|------------|---------------|---------------|-------|-------|-------|-------|-------|
| TF55       | 10.14         | 12.04         | 2.32  | 1.69  | 0.40  | 1.01  | 0.64  |
| TCP1 mouse | —             | 37.85         | 1.45  | 2.25  | 2.30  | 1.56  | 0.84  |
| TCP1 yeast |               | —             | 0.84  | 2.30  | 1.57  | 1.00  | 1.78  |
| groEL      |               |               | —     | 33.74 | 38.80 | 0.64  | 0.39  |
| hsp60      |               |               |       | —     | 24.80 | 2.24  | 1.34  |
| hsp65      |               |               |       |       | —     | -0.39 | -0.97 |
| hsp70      |               |               |       |       |       | —     | 34.86 |

The computer program 'Relate' (Protein Identification Resource; National Biomedical Research Foundation) was used with the Mutation Data Matrix and a segment length of 10 amino acids. Values are given in standard deviation units<sup>37</sup>. A value greater than 7 indicates a significant homology between sequences. Sources of sequences: TF55, Fig. 4a; TCP1b mouse, GENBANK M12899; TCP1 *S. cerevisiae*, GENBANK M21160; groEL *E. coli*, GENBANK S01432; hsp60 *S. cerevisiae*, GENBANK M33301; hsp65 *Mycobacterium tuberculosis*, ref. 38; hsp70 (SSA4) *S. cerevisiae*, GENBANK J05637; dnaK *E. coli*, GENBANK K01298.

at 70 °C reduced its ability to bind Su9-DHFR at 25 °C by ~60% (Fig. 2c). This effect was dependent on TF55 being present during heat denaturation of BSA, however, and was not observed when TF55 was added after denaturation. This suggests that TF55 binds more effectively to proteins as they unfold, rather than to aggregates of already denatured proteins. Furthermore, incubating TF55 with a thermostable alcohol dehydrogenase from *Thermoanaerobium brockii* for 5 minutes at 70 °C does not affect its capacity to bind Su9-DHFR subsequently (Fig. 2c), suggesting that TF55 does not bind native proteins. Neither BSA nor alcohol dehydrogenase was able to prevent the aggregation of Su9-DHFR diluted from 6 M guanidinium chloride in the absence of TF55.

For a more general demonstration of the ability of TF55 to bind unfolded proteins, we used total soluble *Escherichia coli* proteins labelled *in vivo* with L-[<sup>35</sup>S]methionine (Fig. 2d). As expected, binding of these native proteins at 23 °C was negligible (lane 2). When they were heated to 70 °C in the presence of TF55, however, the binding increased substantially (lane 4). This binding was much less pronounced when the *E. coli* proteins were incubated at 70 °C before addition of TF55 (lane 5), corroborating our observation with BSA that the TF55 complex seems to bind proteins as they become unfolded.

Because the binding and release of proteins from chaperones is associated with ATP hydrolysis<sup>16,17,28</sup>, we tested the purified TF55 complex for ATPase activity. At a normal growth temperature for *S. shibatae* (75 °C), 0.45 moles ATP were hydrolysed per mol of complex per second (Fig. 2e), a value similar to that reported for the ATPase activity of the groEL complex at the normal growth temperature of *E. coli*<sup>25,29</sup>. The upper limit of ATPase activity for both complexes correlates with the temperatures at which the complexes dissociate (compare Fig. 2b and e).

The primary structure of TF55 deduced from cloned genomic DNA predicts a hydrophilic polypeptide of 552 amino acids (59.7K) (Fig. 3a). This polypeptide bears a significant relationship along its entire length to a eukaryotic protein, t-complex polypeptide-1 (TCP1; refs 18–21) (Fig. 3b). It has 40% identity and 62% similarity to mouse TCP1 and 36% identity and 59% similarity to *S. cerevisiae* TCP1. Mouse TCP1 is abundantly expressed in developing sperm and has been implicated in the phenomenon of male-specific transmission ratio distortion<sup>18,19</sup>. A more general function of TCP1, however, is suggested by its presence in all other mammalian cell types examined so far<sup>18,19,30</sup>, in *Drosophila melanogaster*<sup>31</sup>, and in *S. cerevisiae*<sup>21</sup>. In *S. cerevisiae*, TCP1 is an essential gene, and a cold-sensitive mutation impairs mitotic spindle formation<sup>21</sup>. At first it was thought that TCP1 was associated with membranes<sup>32</sup>, but a more recent examination indicates that much of the protein is soluble

in the cytosol (V. Lewis, manuscript in preparation). A more general role for TCP1 as a cytosolic molecular chaperone has been proposed, based on its size and limited sequence homology with the bacterial chaperonins<sup>33,34</sup>. We compared the sequences of TF55 and TCP1 with the hsp60 family of chaperonins and with the hsp70 family of chaperones (Table 1). Our comparison confirms the homology between TF55 and TCP1, but indicates no overall homology between either of these molecules and the chaperones tested.

In summary, several lines of evidence suggest that the major heat-shock protein of a thermophilic archaeobacterium functions as a molecular chaperone: it binds unfolded proteins, it prevents their aggregation at high temperature, and it exhibits ATPase activity. Its quaternary structural similarity to the groEL complex, which is known to mediate polypeptide chain folding<sup>13,24,25</sup>, suggests that TF55 may also be involved in *de novo* protein folding and assembly. We have been unable to demonstrate folding activity *in vitro*, however, probably because a cooperating component is required, as in the case of groEL<sup>35</sup>.

The striking similarity between the primary structures of TF55 and TCP1 suggests that these molecules carry out similar functions. The involvement of TCP1 in mitotic spindle formation in yeast<sup>21</sup> and the evidence for a cytoskeleton in archaeobacteria<sup>36</sup> suggest that the two proteins may have a specialized role in cytoskeletal assembly. On the other hand, the predominant synthesis of TF55 during thermal stress and its ability to bind unfolded proteins *in vitro* suggest a more general chaperone function in the archaeobacteria that could include protein folding. Whether TCP1 shares such a general function with TF55 remains to be investigated. □

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# Oncogenic and transcriptional cooperation with Ha-Ras requires phosphorylation of c-Jun on serines 63 and 73

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RECENT advances indicate a link between tumour promoters, transformation, and AP-1 activity<sup>1</sup>. Protein kinase C activation increases AP-1 DNA-binding activity independently of new protein synthesis<sup>2,3</sup>. AP-1 is also stimulated by transforming oncoproteins and growth factors<sup>4-6</sup>. These proteins are thought to participate in a signalling cascade affecting the nuclear AP-1 complex composed of the Jun and Fos proteins<sup>1,7,8</sup>. Because c-Jun is the most potent transactivator in the AP-1 complex<sup>9-12</sup> and is elevated in Ha-ras-transformed cells, in which c-Fos is downregulated<sup>13,14</sup>, we focused on it as a potential target. c-Jun could convert input from an oncogenic signalling cascade into changes in gene expression. Indeed, transformation of rat embryo fibroblasts by c-Jun requires an intact transcriptional activation domain<sup>15</sup> and cooperation with oncogenic Ha-ras<sup>16</sup>. Expression of oncogenic Ha-ras augments transactivation by c-Jun and stimulates its phosphorylation<sup>14</sup>. Here we describe the mapping of the Ha-ras-responsive phosphorylation sites to serines 63 and 73 of c-Jun. Site-directed mutagenesis indicates that phosphorylation of these serines is essential for stimulation of c-Jun activity and for cooperation with Ha-ras in oncogenic transformation.

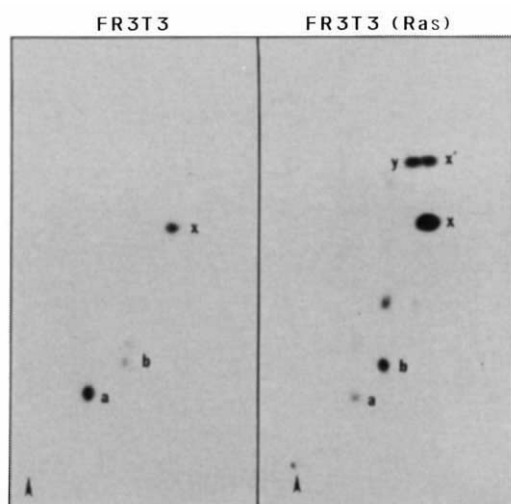


FIG. 1 c-Jun is hyperphosphorylated in Ha-ras-transformed cells. Subconfluent cultures of FR3T3 and Ha-ras-transformed FR3T3 cells were metabolically labelled for 2.5 h with either <sup>32</sup>P-orthophosphate (for phosphopeptide mapping) or <sup>35</sup>S-methionine (to normalize the levels of protein used for phosphopeptide mapping). Two-dimensional tryptic phosphopeptide analysis was done on equivalent amounts c-Jun protein isolated by immunoprecipitation as described<sup>14</sup>. The FR3T3 and FR3T3(Ras) maps were exposed for 8 and 4 days, respectively, to normalize for the difference in <sup>32</sup>P-orthophosphate uptake between nontransformed and Ha-ras-transformed NIH3T3 cells<sup>14</sup>. The letters a and b refer to the tri- and diphosphorylated forms of the C-terminal peptide<sup>17</sup>. The origin is marked by an arrowhead.

Transient coexpression of oncogenic Ha-Ras with c-Jun leads to hyperphosphorylation of two sites, x and y (ref. 14). To extend these findings to stable transformants, we isolated c-Jun from normal Ha-ras-transformed FR3T3 cells, labelled with <sup>32</sup>P-orthophosphate and analysed it by two-dimensional tryptic peptide mapping. Figure 1 demonstrates that Ha-ras transformation results in a marked increase in phosphopeptides x and y. (As shown in Fig. 2, phosphopeptide x' is derived from x.) Ha-ras transformation also causes dephosphorylation of one or more C-terminal sites that give rise to phosphopeptides a and b, which are the tri- and diphosphorylated forms of the same tryptic peptide, respectively<sup>17</sup>. Phosphorylation of these sites inhibits DNA binding<sup>17</sup>.

Previous work suggested that phosphorylation of sites x and y modulates c-Jun activity<sup>14</sup>. Therefore, we mapped the precise location of these sites. The x and y peptides reside in the first 87 amino acids of c-Jun<sup>14</sup> and contain phosphoserines<sup>17</sup>. Tryptic peptide maps of <sup>32</sup>P and <sup>35</sup>S doubly and singly labelled c-Jun indicated that neither x nor y contained methionine (our unpublished results). We also found that v-Jun and a JunB/c-Jun hybrid, containing the N-terminal half of JunB, respond to Ha-Ras<sup>14</sup> and therefore may contain similar sites. Only two tryptic peptides derived from the N-terminal region of c-Jun contain serines and no methionine, and are relatively conserved between c-Jun, v-Jun and JunB: NSDLLTPDVGLLK and LASPELER. These peptides were also selected as candidates for y

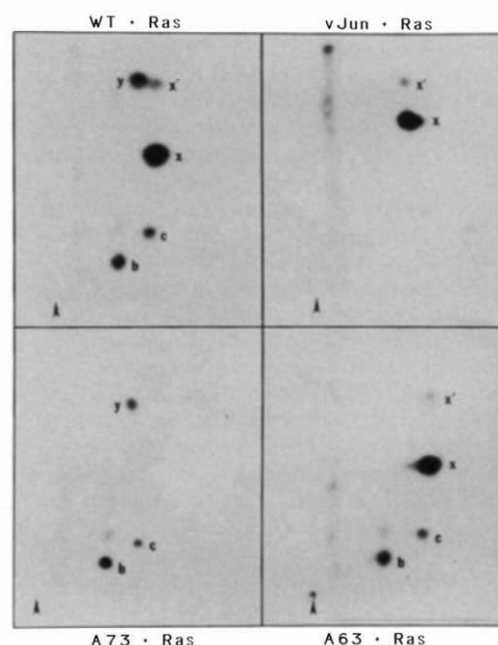
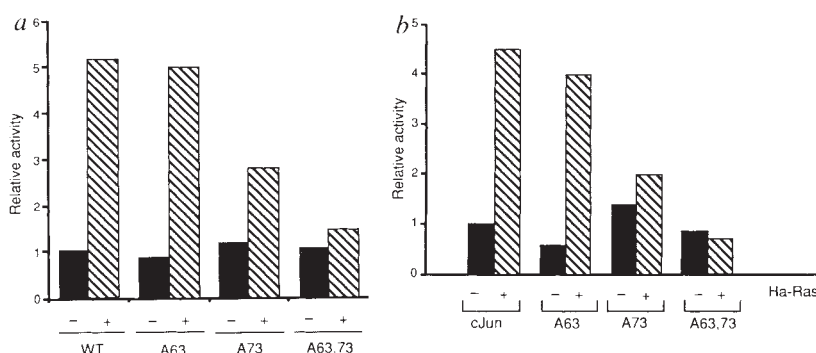


FIG. 2 Identification of the x and y phosphorylation sites. The assignment of the x and y phosphorylation sites to serines 63 and 73, respectively, was confirmed by two-dimensional maps of wild-type and mutant c-Jun proteins. F9 cells were transfected with 10  $\mu$ g of expression vectors encoding wild-type (WT) c-Jun and the Ala 63 and Ala 73 substitutions as well as v-Jun. pZIPNeoRas(leu61) (5  $\mu$ g) was included to enhance detection of the x and y phosphopeptides. Cells were placed into fresh medium 8 h after transfection and labelled for 3 h with <sup>32</sup>P-orthophosphate, 20 h after transfection. c-Jun proteins were purified by immunoprecipitation and subjected to two-dimensional phosphopeptide analysis. Maps were exposed to X-ray film for 9 days. The letters b and c refer to the di- and monophosphorylated forms of the C-terminal peptide<sup>17</sup>. The origin is marked by an arrowhead. METHODS. To mutate the codons specifying serines 63 and 73 of c-Jun the *XhoI*-*PstI* restriction fragment of RSV-c-Jun was subcloned between the *Bam*HI and *PstI* sites of M13mp18 and single-stranded DNA from the resulting construct was used as a template for site-directed mutagenesis using the Amersham site-directed mutagenesis system, as recommended by the manufacturer. The mutagenic primers 5'-CAGCCCCACGACCGAGCGG-TGAGGAGGTC-3' and 5'-GCGCTCCAGCTCCGAGCCGCCAGCTTGAG-3' were used to convert Ser 63 to Ala and Ser 73 to Ala, respectively.



FIG. 3 Effect of phosphorylation site mutations on transactivation. *a*, NIH3T3 cells were cotransfected with 10  $\mu$ g -90GHF1-CAT( $\Delta$ AP-1) reporter and 5  $\mu$ g RSV-cJ/GHF1, RSV-cJ/GHF1(Ala 63), RSV-cJ/GHF1(Ala 73) or RSV-cJ/GHF1 (Ala 63, 73) with or without 5  $\mu$ g pZIP-NeoRas(leu61), as indicated. One unit of relative activity is equivalent to threefold activation by RSV-cJ/GHF1. The averages of three experiments are shown. *b*, F9 cells were cotransfected with 2  $\mu$ g of the AP-1 responsive -73Col-CAT reporter<sup>2</sup> and 1  $\mu$ g of either RSV-c-Jun, RSV-c-Jun(Ala 63), RSV-c-Jun(Ala 73) or RSV-c-Jun(Ala 63, 73) with or without 1  $\mu$ g pZIPNeoRas(leu61), as indicated. One unit of relative activity is equivalent to 20-fold activation of -73Col-CAT expression by RSV-c-Jun. The averages of three experiments are shown.



and x, respectively, by a computer program that predicts the mobilities of tryptic peptides<sup>18</sup>. The two peptides were chemically synthesized, phosphorylated *in vitro* (using a nuclear extract of Ha-ras transformed FR3T3 cells), and found to co-migrate with the x and y phosphopeptides derived from metabolically labelled c-Jun (A. N. Lin and T.S., unpublished results). As x and y phosphorylation is coordinately regulated, the most likely targets for phosphorylation were Ser 63 and Ser 73 because they are in very similar sequences, whose consensus is LT/ASPD/EV/L.

To determine whether these residues were targets for phosphorylation, we substituted them with alanines using site-directed mutagenesis. Expression vectors encoding wild-type (WT) c-Jun and each of the two Ser→Ala substitutions were cotransfected into F9 cells with the Ha-Ras expression vector. The proteins were metabolically labelled with <sup>32</sup>P, immunopurified and analysed by tryptic peptide mapping. The Ala 63 substitution resulted in disappearance of phosphopeptide y, whereas the Ala 73 substitution resulted in disappearance of phosphopeptide x and the minor phosphopeptide x' (Fig. 2). Because x' has the same electrophoretic mobility as x but is more hydrophobic and its relative amount was somewhat variable, it is probably derived by alkylation of peptide x during sample handling (T. Hunter, personal communication). None of these mutations had any effect on the level of c-Jun, its stability (Fig. 4b), or phosphorylation of the nonmutagenized C-terminal sites.

The sequence of the x peptide is fully conserved in v-Jun, whereas the sequence of the y peptide is not<sup>19</sup>. Tryptic peptide maps of <sup>32</sup>P-labelled v-Jun reveal the presence of a phosphopeptide with identical mobility to x, but the y phosphopeptide is missing (Fig. 2). These results give further support to the assignment of serines 63 and 73 as the y and x sites, respectively. As described earlier, v-Jun is not phosphorylated on the C-terminal sites<sup>17</sup>.

Because the DNA-binding activity of c-Jun is regulated both by phosphorylation<sup>17</sup> and association with Fos<sup>20,21</sup>, we examined the effect of the mutations on activity of a c-Jun/GHF1 hybrid<sup>14</sup>. This ensured that the effects were due to phosphorylation of serines 63 and 73 in c-Jun's activation domain, as GHF-1 itself is not phosphorylated under these conditions<sup>14</sup>. Although the Ala 63 substitution had a marginal effect on either the basal activity of the hybrid or on its response to Ha-Ras in NIH3T3 cells, the Ala 73 substitution partially inhibited its response to Ha-Ras, while slightly increasing its basal activity (Fig. 3a). c-Jun/GHF1(Ala 63/73) was no longer responsive to Ha-Ras but its basal activity was similar to that of wild-type c-Jun/GHF1. This protein was expressed at the same level as the wild-type hybrid (data not shown). Similar results were obtained by analysis of wild-type and mutant c-Jun proteins having single and double amino-acid substitutions in the absence and presence of Ha-Ras in F9 cells (Fig. 3b). Therefore phosphorylation of both serines is required for a maximal stimulation of c-Jun activity by Ha-Ras.

c-Jun cooperates with Ha-ras in oncogenic transformation<sup>16</sup>. As transactivation by c-Jun is required for transformation<sup>15</sup>, this

oncogenic cooperation probably requires enhancement of c-Jun activity by an Ha-Ras-activated phosphorylation cascade. Therefore we examined the ability of wild-type c-Jun and c-Jun(Ala 63/73) to cooperate with Ha-ras in rat embryo fibroblast transformation. Although cotransfection of two different expression vectors encoding wild-type c-Jun markedly potentiated foci formation by activated Ha-ras, no significant increase in transformation was observed on cotransfection of Ha-ras with the same expression vectors encoding c-Jun(Ala 63/73) (Fig. 4a). Despite its inability to cooperate with Ha-ras, c-Jun(Ala 63/73) was expressed as efficiently as wild-type c-Jun (Fig. 4b).

These experiments demonstrate that activated Ha-Ras stimulates c-Jun activity by inducing hyperphosphorylation of its activation domain on serines 63 and 73. Substitution of serines 63 and 73 by alanines inhibits the response of c-Jun to Ha-Ras without affecting its basal activity. These substitutions do not affect expression of c-Jun or its phosphorylation on negative regulatory sites, adjacent to its DNA-binding domain. Most

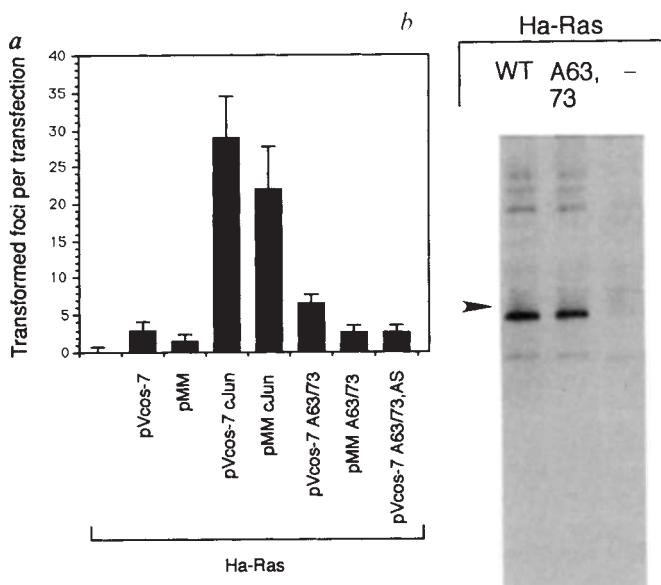


FIG. 4 Serines 63 and 73 are required for cooperation with Ha-Ras. *a*, Subconfluent cultures of rat embryo fibroblasts were prepared and transfected as described previously<sup>15,16</sup>. The plasmids used included: pEJ6.6, an activated Ha-ras gene (kindly provided by R. Weinberg); pVcos-7, a eukaryotic expression vector driven by a Moloney leukaemia virus long terminal repeat; pMM, a eukaryotic expression vector driven by a mouse metallothionein 1 promoter<sup>15</sup>. pEJ6.6 was cotransfected with either expression vector harbouring wild-type c-Jun or the c-Jun(A63, 73) mutant in the sense orientation. Transformed foci were identified after two weeks and scored as foci per transfection (shown with s.d. of two separate experiments done in triplicates). Control transfections included Ha-ras alone, Ha-ras plus either vector and Ha-ras plus c-Jun(A63/73) in the antisense orientation. *b*, c-Jun expression vector containing either WT c-Jun or the c-Jun(Ala 63/73) was transiently transfected with pZIPNeoRas(leu61). Cells were labelled 20h after transfection with <sup>35</sup>S-methionine for 2 h. Immunoprecipitates were analysed by SDS-PAGE as described<sup>14</sup>.

importantly, substitution of serines 63 and 73 with alanines abolishes oncogenic cooperation between c-Jun and Ha-Ras. Although extensively studied, the mechanism of oncogenic cooperation is not understood<sup>22,23</sup>. In this case, cooperation requires post-translational enhancement of c-Jun activity by Ha-Ras. This conclusion is supported by the finding that a dominant negative c-Jun induces reversion of *ras*-transformed cells<sup>24</sup>. This provides a biochemical explanation for at least one case of oncogenic cooperation and suggests that a major pathway for transformation by Ha-*ras* involves the activation of c-Jun by a phosphorylation cascade.

Although the target with which the c-Jun activation domain interacts is not identified, these results suggest that upon phosphorylation the activation domain can interact with its target more efficiently. It is also possible that the phosphorylated activation domain interacts with an additional target, with which it does not interact before phosphorylation. These positive interaction models are supported by the results of the mutagenesis experiments. Substitution of serines 63 and 73 with alanines had no effect on basal activity of c-Jun but prevented its enhancement

by Ha-Ras. Several other transcription factors are also regulated by phosphorylation. Most relevant is CREB, which is converted by phosphorylation on Ser 133 by protein kinase A from a transcriptionally inert protein to a potent transactivator<sup>25</sup>. With the exception that the non-phosphorylatable c-Jun activation domain possesses considerable basal activity, its mode of regulation is similar to that of CREB. It is possible that this mode of regulation is common to other members of the bZIP family of transcriptional regulators<sup>26</sup>, especially those proteins whose overall organization is similar to CREB and c-Jun.

In addition to stimulating phosphorylation of the c-Jun activation domain, expression of Ha-Ras leads to dephosphorylation of at least one of three inhibitory phosphorylation sites next to its DNA-binding domain. Treatment of fibroblasts and epithelial cells with phorbol ester also leads to dephosphorylation of the inhibitory sites, but unlike Ha-Ras, has only a small stimulatory effect on phosphorylation of serines 63 and 73 (ref. 17). It would be of interest to determine whether the limited effect of phorbol ester on c-Jun phosphorylation in fibroblasts is related to its partial mitogenic activity. □

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## Cloning and expression of the essential gene for poly(A) polymerase from *S. cerevisiae*

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**POLY(A) polymerase is essential for the maturation of messenger RNA, adding tracts of adenosine residues to the 3' end of precursor RNA generated by endonucleolytic cleavage. This mechanism of mRNA 3' processing seems to be similar in yeast and in higher eucaryotes<sup>1</sup>, although there are differences in the recognition signals in the pre-mRNA<sup>2</sup>. Here we describe the cloning of the gene for yeast poly(A) polymerase. The enzyme is encoded by a single and essential gene located near the centromere on the left arm of chromosome 11. Poly(A) polymerase purified from recombinant *Escherichia coli* has the same physical and biochemical properties as the yeast enzyme. The yeast poly(A) polymerase shares features of sequence with its mammalian homologue<sup>3,4</sup>.**

Purified yeast poly(A) polymerase (PAP)<sup>5</sup> was digested with trypsin and the amino-acid sequence<sup>6</sup> of five HPLC-purified peptides was determined. The first 14 amino acids from one peptide were used for the design of two DNA oligonucleotides and a PAP-specific DNA fragment was amplified by PCR (Fig. 1a). Two genomic clones were isolated from a yeast plasmid library using this probe<sup>7</sup> (see legend to Fig. 1). The coding region for PAP was located by Southern blotting on a 2,286-base

pair(bp) *XhoI-HindIII* fragment common to both clones. This fragment contained an open reading frame encoding 568 amino acids, including all sequences obtained from the tryptic peptides (Fig. 1b). Its predicted relative molecular mass ( $M_r$ ) of 64,551 was in good agreement with the estimate of 63,000 for purified PAP<sup>5</sup>.

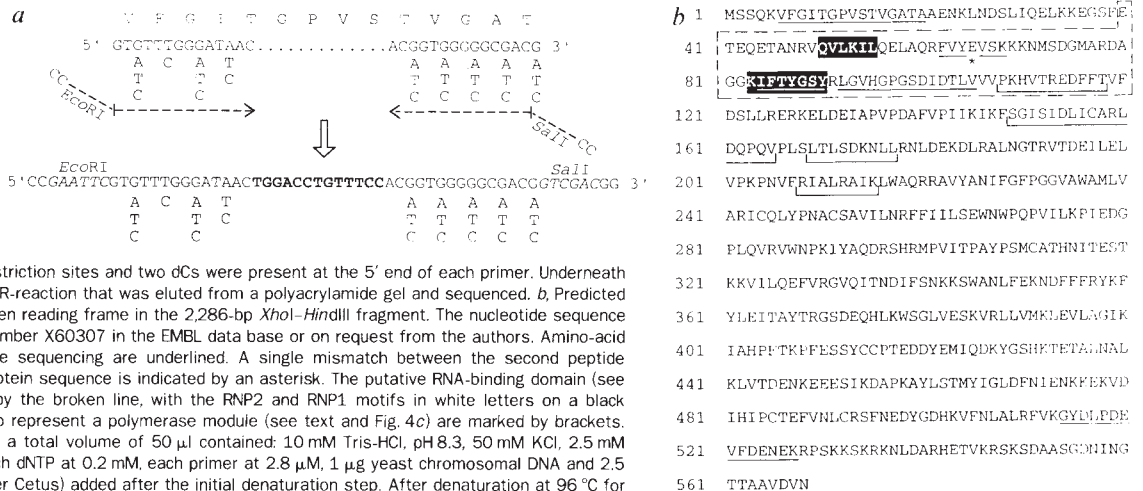
The gene (*PAP1*) was expressed in *Escherichia coli* under the control of the phage T7 promoter<sup>8</sup>. The specific activity of PAP in a crude extract of the recombinant *E. coli* strain was 120-fold higher than that of a control extract. On purification (Fig. 2), a protein of the size expected comigrated with PAP activity through all steps. Chromatographic properties, electrophoretic mobility (Fig. 2b; compare lanes 5 and 10) and specific activity ( $1.3 \times 10^6$  U per mg) of the recombinant enzyme were indistinguishable from authentic PAP<sup>5</sup>.

Southern blot hybridization of yeast genomic DNA cut with different enzymes showed that *PAP1* is a single-copy gene in the haploid genome (Fig. 3a). The yeast chromosome contains the same *HindIII* fragment that is present in the isolated clone. We constructed a strain heterozygous for a deletion in the *PAP1* gene<sup>9</sup>: 84% of the *PAP1* coding region was replaced by a *LEU2* gene insertion on a plasmid (Fig. 3a, lower panel). A linearized fragment containing this *LEU2* marker and the *PAP1* flanking region was used to transform a diploid leucine auxotrophic yeast strain. *Leu*<sup>+</sup> transformants were selected and analysed by Southern blotting (Fig. 3a). All seven transformants analysed contained one disrupted *PAP1* allele and one wild-type allele. The isolates were sporulated and tetrads dissected. All viable spores were leucine auxotrophs and in no case did more than two of the four separated spores form colonies. The deletion could be rescued when *PAP1* was supplied on a centromeric plasmid (data not shown). Thus, *PAP1* is indispensable for cell growth.

In a data bank search, the region upstream of the *PAP1* gene



FIG. 1 *a*, Primer design for the amplification by polymerase chain reaction (PCR) of a *PAP1*-specific DNA fragment (top). (The peptide sequence is in single-letter code.) The sense orientation of possible corresponding DNA sequences is shown. The primers (completely degenerate) are indicated by arrows pointing 5' to 3'. Restriction sites and two dCs were present at the 5' end of each primer. Underneath is shown the product of the PCR-reaction that was eluted from a polyacrylamide gel and sequenced. *b*, Predicted amino-acid sequence of the open reading frame in the 2,286-bp *XhoI*-*HindIII* fragment. The nucleotide sequence is available under accession number X60307 in the EMBL data base or on request from the authors. Amino-acid sequences obtained by peptide sequencing are underlined. A single mismatch between the second peptide sequence and the predicted protein sequence is indicated by an asterisk. The putative RNA-binding domain (see text and Fig. 4b) is indicated by the broken line, with the RNP2 and RNP1 motifs in white letters on a black background. Motifs proposed to represent a polymerase module (see text and Fig. 4c) are marked by brackets. METHODS. The PCR reaction in a total volume of 50  $\mu$ l contained: 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.5 mM MgCl<sub>2</sub>, 0.01% Triton X-100 each dNTP at 0.2 mM, each primer at 2.8  $\mu$ M, 1  $\mu$ g yeast chromosomal DNA and 2.5 units of Ampli Taq (Perkin Elmer Cetus) added after the initial denaturation step. After denaturation at 96 °C for 5 min, the reaction was carried out for 30 cycles at 94 °C for 15 s, 48 °C for 15 s and 72 °C for 1 min. The fragment obtained was purified and labelled with <sup>32</sup>P. A plasmid library containing yeast genomic fragments in YE13 (ref. 7) was screened by standard methods<sup>23</sup>. Sequencing was by the dideoxynucleotide chain-termination method<sup>24</sup> using Sequenase (US Biochemicals) on nested deletions generated with exonuclease III (ref. 25). Both strands of the entire gene were sequenced.



was found to be identical with the sequence upstream of *VPS1* (or *SPO15*; ref. 10), a putative GTP-binding protein<sup>11</sup> essential for meiosis<sup>12</sup>. This gene is located on the left arm of chromosome 11, close to the centromere<sup>12</sup>. The published restriction map of the genomic *VPS1* upstream region<sup>11</sup> also fits our data. The initiation codons of *PAP1* and *VPS1* are 510 base pairs apart and transcription is divergent. The organization of both genes is depicted in Fig. 3b (lower panel).

Comparison of the amino-acid sequence of PAP to the SWISS-PROT data base did not reveal long stretches of similarity to other known proteins. But 47 per cent of the 400 N-terminal amino acids of yeast PAP are identical to those of the mammalian enzyme. The C-terminal region is unrelated (Fig. 4a). Therefore, the catalytic function of both polymerases may reside in a conserved N-terminal domain, whereas the diverged C terminus could be responsible for the interaction with the cleavage and polyadenylation factor, CPF (ref. 13), a factor so far identified only in mammals. This may explain why the yeast enzyme cannot substitute for its mammalian counterpart in an *in vitro* polyadenylation assay (W.K., unpublished results).

The N-terminal region of the mammalian polymerase<sup>3,4</sup> shows

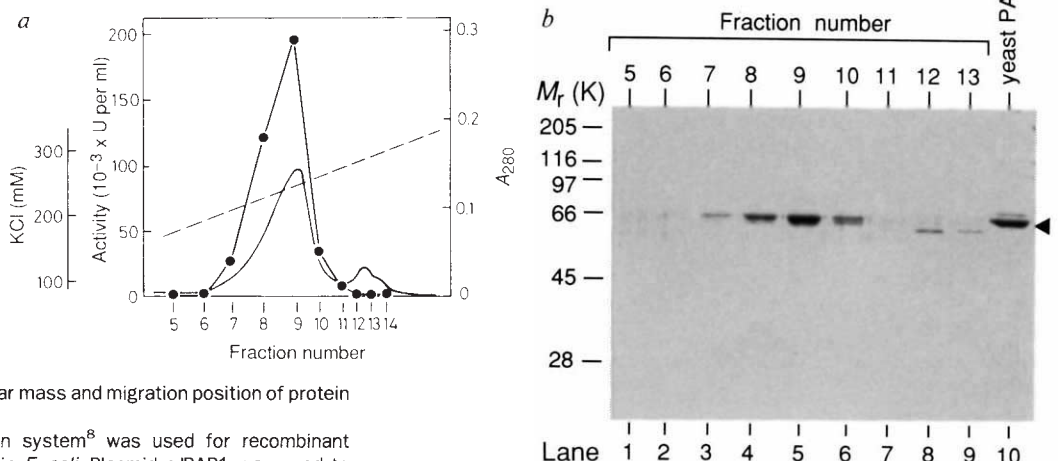
some similarity with the RNA-binding domain of RNA-binding proteins<sup>14-16</sup>. The similarity, although less, is still detectable in the yeast enzyme (Fig. 4b). Moreover, secondary structure prediction of the putative RNA-binding domains of the yeast and mammalian PAP (M. Görlach, E. Matunis and G. Dreyfuss, personal communication) agrees reasonably well with the structure predicted for the RNA-binding domains present in the other members of this class of proteins. Nevertheless, it is surprising to find an RNA-binding domain in poly(A) polymerases. Most other members of the protein family having the RNA-binding domain form stable RNA-protein complexes (for example, the small nuclear RNP particles involved in splicing). By contrast, yeast and mammalian poly(A) polymerases bind to their RNA primers transiently. This is reflected by a high  $K_m$  and a distributive interaction<sup>5,17</sup>.

It has been proposed<sup>3</sup> that mammalian PAP contains the so-called polymerase module, a weakly conserved sequence present in many nucleic acid-synthesizing enzymes<sup>18,19</sup>, which might represent a universal polymerase core<sup>20-22</sup> (Fig. 4c). Comparison of the amino acid sequence of yeast and mammalian PAP indicates that the assignments of motifs within the putative

FIG. 2 Mono-S chromatography of recombinant PAP from *E. coli*.

*a*, The protein elution profile (full line) was monitored by absorbance at 280 nm ( $A_{280}$ ). The salt gradient is indicated by the dashed line. Activity (filled circles) was assayed as described<sup>5</sup>. Fraction numbers are indicated. *b*, Electrophoresis of Mono-S fractions on a 10% polyacrylamide-SDS gel. Fraction numbers are indicated above each lane (lanes 1-9); lane 10, poly(A) polymerase purified from yeast (marked by an arrowhead). The relative molecular mass and migration position of protein markers is indicated on the left.

METHODS. *a*, A T7 overexpression system<sup>8</sup> was used for recombinant expression of poly(A) polymerase in *E. coli*. Plasmid pJAP1 was used to transform BL21(DE3) pLysS. pJAP1 contains the coding region of *PAP1* and 100 bp of untranslated trailer cloned into the T7 transcription/translation vector pJC10 (ref. 26). This construct allowed the expression of poly(A) polymerase from its natural initiation codon. Details of the procedures are available upon request. Extracts were prepared from 2-litre cultures as described<sup>8</sup>, with minor modifications. The extract was diluted to 50 ml to



give final concentrations of 100 mM KCl, 50 mM Tris-HCl, pH 8.0, 0.5 mM DTT, 1 mM EDTA, 10% glycerol, and loaded onto a 100-ml DEAE-Sepharose column equilibrated in the same buffer. The flow-through that contained all the activity was collected and the enzyme was purified further by chromatography on hydroxyapatite and Mono S essentially as described<sup>5</sup>. About 1.8 mg of purified protein was obtained with an overall recovery of 35%.

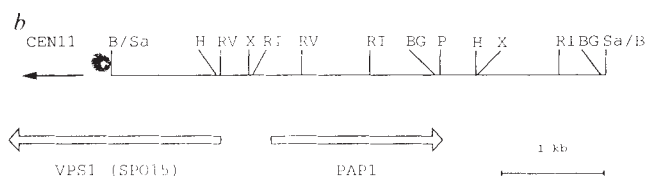
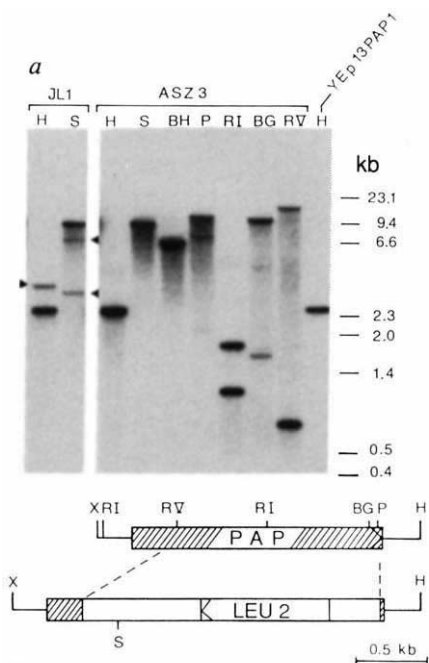
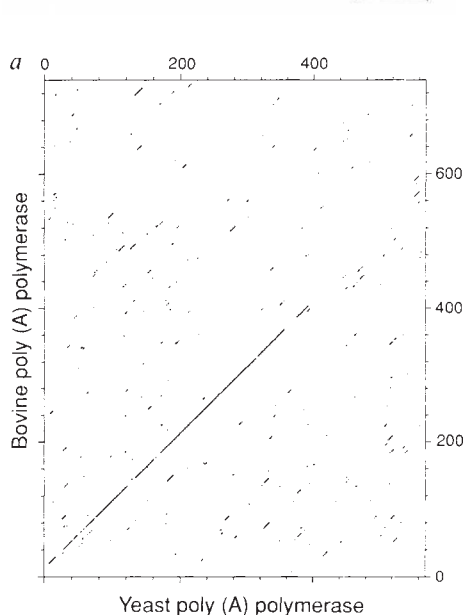


FIG. 3 *a*, Southern blot analysis of chromosomal DNA of wild-type ASZ3 (*a/a ade2-1/ade2-1 his3-11, 15/his3-11, 15 leu2-3, 112/leu2-3, 112 trp1-1/trp1-1 ura3/ura3 can1-100/can1-100*; A. Stotz, personal communication), JL1 (*PAP1/pap1::LEU2*) and plasmid DNA of the isolated clone YEp13PAP1. DNA was digested with restriction enzymes as indicated above each lane and probed with the 2,286-bp *XhoI*-*HindIII* fragment covering the *PAP1* coding region (lower panel). Restriction enzyme cutting sites are indicated by B (*Bam*HI), Sa (*Sau*3AI), H (*Hind*III), RV (*Eco*RV), X (*Xho*I), RI (*Eco*RI), BG (*Bgl*II) and P (*Pvu*II). The migration of DNA size markers is indicated to the right (in kb). Arrowheads point to new restriction fragments resulting from the insertion of the *LEU2* marker. Underneath is shown (top) the probe used for hybridization, and (bottom) the fragment used for disruption of one *PAP1* allele. The *PAP1* region that was replaced by *LEU2* is indicated by the broken lines. *b*, Restriction map of the isolated clone YEp13PAP1 containing the *PAP1* gene. Open arrows below the map indicate the direction of transcription and the extension of *PAP1* and *VPS1* (also called *SPO15*). The relative position of centromere 11 close to the 3' end of *VPS1* is indicated (thin arrow).

METHODS. Chromosomal yeast DNA was prepared as described<sup>27</sup>. Standard techniques were used for Southern blotting<sup>23</sup>. For the construction of JL1, the yeast strain ASZ3 was transformed with pPAPΔLEU (linearized with *XhoI* and *HindIII*) by electroporation<sup>28</sup>. pPAPΔLEU was constructed by deleting most of *PAP1* with *HincII* and *SnaBI* and replacing this region with the 2.1-kb *HpaI* fragment of YEp13 which contains the entire *LEU2* gene.



|              | RNP2                                                      |  | RNP1                  |
|--------------|-----------------------------------------------------------|--|-----------------------|
| Yeast PAP :  | QVLK(1)E(2)E(3)E(3)VS(1)K(12)G(0)R(1)TYGS(8)S(8)P(1)H     |  | R(1)TYGS(8)S(8)P(1)H  |
| Bovine PAP : | FILEK(1)N(0)E(3)W(3)IS(1)S(12)G(0)R(1)E(3)S(8)A(8)P(1)H   |  | R(1)E(3)S(8)A(8)P(1)H |
| Consensus :  | LFVGNL(5)E(2)L(3)F(2)FG(1)I(6-12)K(2)KGFGFVXF(6)A(8)L(1)G |  | R(1)E(3)S(8)A(8)P(1)H |
|              | IYIKG D Y V R                                             |  | R YA Y I              |

|              | Motif A             | Motif B                   | Motif C                  | Motif D   |
|--------------|---------------------|---------------------------|--------------------------|-----------|
| Yeast PAP :  | PK(1)VT(1)E(2)E(31) | SG(3)D(1)I(1)ARLD(2)QV(3) | L(1)LS(1)LL(29)R(2)L(3)R |           |
| Bovine PAP : | PK(1)VD(1)S(2)E(31) | D(3)D(1)L(1)ARLA(2)TI(3)  | L(1)LR(1)LL(29)R(2)L(3)R |           |
| Consensus :  | hh(1)hD(1)S(2)FD    | SG(3)S(1)h(1)NShh(2)hh    | h(1)hGDD(1)hh            | G(2)h(4)K |
|              | * T Y               | T                         | **                       | *         |

FIG. 4 Structural features of poly(A) polymerase. *a*, Sequence comparison of yeast and bovine PAP (taken from ref. 4) by a diagonal blot<sup>29</sup>. The comparison was run with a window size of twelve and a minimal score of eight amino acids. The homology ends after about 400 amino acids. *b*, Alignment of yeast and bovine PAP with the RNA-binding domain consensus (M. Görlich, E. Matunis and G. Dreyfuss, personal communication). Only conserved residues are indicated. Distances between residues are given in brackets. The highly conserved RNP1 and RNP2 motifs are boxed. Amino acids that fit the consensus are indicated by white letters on a black background, conservative exchanges are bold. *c*, Alignment of yeast and bovine PAP to the polymerase module<sup>19</sup> (consensus taken from ref. 30). Amino acids are labelled as in *b*, hydrophobic residues are labelled with h and bold letters indicate a fit to hydrophobic residues. Key residues that are invariant in proteins belonging to this family are marked by an asterisk. The only known deviation for DD in motif C is DN<sup>19</sup>. The alignment was done according to Raabe *et al.*<sup>3</sup>.

polymerase module are probably not correct. Some of the invariant amino acids (marked with asterisks in Fig. 4c) are not conserved in the yeast poly(A) polymerase, such as the Asp in motif A and the Asp-Asp in motif C. Also, the distance between motifs B and C is much too short to make sense in terms of protein structure. It is possible to find regions in the sequence of yeast and mammalian PAP that have a better match to a

potential polymerase module (O. Poch, personal communication). However, we think that the existence of a polymerase module should be demonstrated by the analysis of mutants and by determining the protein structure.

The cloning of the gene for yeast poly(A) polymerase should also lead to a better understanding of the mechanism of 3' processing of mRNA and the function of poly(A) tails. □

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